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BIOREACTORS: A Humane Alternative to Mab Production from Mouse Ascities

by

Trivikram Chebrolu

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Pharmaceutical Sciences

Faculty of Pharmacy and Pharmaceutical Sciences

Edmonton, Alberta
Fall 2002

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Submitted to the Faculty of Graduate studies and Research on 9th - AUGUST - 2002

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Abstract

Monoclonal antibodies (Mabs) have wide applications as research tools, diagnostic tools and therapeutic agents. For all these applications, the primary methods available to generate research quantities of Mabs (10mg-100mg) are static tissue culture flasks and ascities fluid from mice. With the advent of various commercially available *in-vitro* systems for production of highly concentrated Mab, animal ethics committees have pressed for a review (and in some instances banning) of the practice of ascities production in mice. In this study, we compared the relative Mab yields of a hybridoma secreting mouse monoclonal antibodies in different systems. To evaluate the production of monoclonal antibodies, four different media were used in a combination with four different bioreactors based on different principles of construction and operating mechanism. The simple I-Mab bag system with DMEM-10 % FBS appears to be the best alternative to ascities production. An innovative approach of perfluorocarbon supplementation for better oxygenation of the bioreactor was explored resulting in increased Mab production.

A generic ELISA was optimized to estimate the mouse Mabs, based on the popular sandwich assay design. Twelve different combinations of four solid phases and three solution phase enzyme conjugate reporters were evaluated to select one successful format of SPIA. This SPIA incorporated a goat anti-Fc specific solid phase capture antibody and a solution phase goat anti-mouse HRPO conjugate as the detecting reagent. The mouse IgG assay developed had a dynamic range from nano gram to micro gram for estimation of mouse IgG from raw culture supernatants.

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List of abbreviations and symbols

A_{280}	Absorbance at 280nm
Ab	Antibody
Ag	Antigen
AP	Alkaline phosphatase
ATCC	American tissue culture collection
AWA	Animal welfare act
BD	Becton Dickinson
BSA	Bovine serum albumin
bsMab	Bispecific Monoclonal antibody
CD	Cluster of differentiation
CFC	Chlorofluorocarbons
CL-1000	Cell line 1000mL culture flask
CNBr	Cyanogen bromide
CTL	Cytotoxic T Lymphocytes
DMEM	Dulbecco modified eagle medium
DNA	De-oxy ribonucleic acid
DND	Department of defense
DRES	Defense Research & Establishment Suffield
EBV	Epstein-Barr Virus
EIA	Enzyme immuno assay
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorting

FBS	Fetal bovine serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
GAM	Goat anti mouse
HAMA	Anti human mouse antibody
HAT	Hypoxanthene, Aminopterin, Thymidine
Hb	Haemoglobin
HER2	Herceptin receptor 2
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
HRPO	Horse Radish Peroxidase
HSP	Heat shock protein
HSUS	Humane society of the united states
IACUC	Institutional animal care and use committees
I-131	Iodine 131
IgG	Immunoglobulin
I-Mab	Commercial brand name of gas permeable bag from DCL diagnostics
KDa	Kilo daltons
Mab	Monoclonal antibody
MEM	Minimum essential medium
MW	Molecular weight
NASA	National Aeronautics and Space Administration
NK	Natural Killer
NMR	Nuclear Magnetic Resonance

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
PFC	Perfluorocarbon
PSG	Pencillin-streptomycin and glutamine
RES	Reticulo endothelial system
RIA	Radio immuno assay
RNA	Ribo nucleic acid
RPMI	Roseland primrose medical institute
RSV	Respiratory syncytial virus
SCID	Severe combined immuno deficiency
SDS	Sodium dodecylsulfate
SFM	Serum free medium
SPIA	Solid phase immunoassay
STP	Standard temperature and pressure
TC-tech	Tissue culture- gas permeable bags from TC-tech company
TMB	Tetra methyl benzidine
TNF	Tumor necrosis factor
USDA	United states department of agriculture
UV	Ultra violet
VEE	Venezuela equine encephalitis
WEE	Western equine encephalitis

CHAPTER-I

Introduction

1.1 Antigens & Antibodies

Antibodies or immunoglobulins are a group of glycoproteins present in the serum and tissue fluids of all mammals. They are produced by the immune system in response to immunogenic foreign molecules called antigens and bind specifically to the antigen that induces their formation. The antibody can be regarded as a bifunctional molecule, wherein one part binds the antigen and the other part may bind to the receptors on phagocytic cells or to the complement components that can then eliminate the antigen. An antigen is a term used for a molecule which elicits a specific antibody. Generally it is a large MW foreign substance and can be soluble or may be part of a cell, bacteria, virus, or other pathogen. In the case of autoimmune diseases, an antigen can be a molecule belonging to one of the body's own cells.

1.2 Polyclonal antibodies

Traditionally antibodies were produced by immunizing suitable animals, for example rabbits, horses, cows, goats etc depending on how much antibody was required. The antibodies produced in this way were collected by bleeding the animal and separating the serum from the collected blood. The serum containing the desired antibody contains many other components that might interfere with the desired antibody-antigen reaction. Difficulty arises, however, from the fact that sera produced from animals invariably contain many different antibodies reactive with a wide range of antigens. This

sera is called polyclonal sera as they contain many different antibodies produced by many different clones of antibody producing cells (B cells). The polyclonal serum pool of antibodies can pose problems when specific antibodies are required in high titres for particular experiments or for clinical therapy. Thus the production of homogeneous antibodies of the required specificity has been a long-standing goal in biomedical research.

1.3 Hybridoma technology-Basis of Mabs

Homogeneous antibodies were first isolated from B cell tumors (Kohler.G and Milstein, C 1975). B cells are lymphopoietic cells, which differentiate in response to antigens to become plasma cells. The plasma cells secrete antibodies. All the antibodies secreted by a clone of B cells are identical, so these tumor cells provide a good source of homogeneous antibody. The First Mabs were of mouse origin and based on the Nobel Prize-winning research of Kohler and Milstein in 1975. The two scientists were studying B cells, or B-lymphocytes, which produce millions of different antibodies to detect, bind, and help eliminate foreign molecules and pathogens from the body. At the time, it wasn't certain whether a single B cell could produce a multitude of antibodies capable of binding different antigens or whether antibodies made by a B cell always bound the same antigen. The two scientists discovered that the latter possibility was true: All antibodies produced by a single B cell have identical binding properties. This discovery meant that, in principle, all one needed to do to produce large amounts of a single, desirable antibody was to isolate and then culture a single B cell that produced it. The cultured cells, all derived from one parent, cell, or clone, would all secrete the same antibody

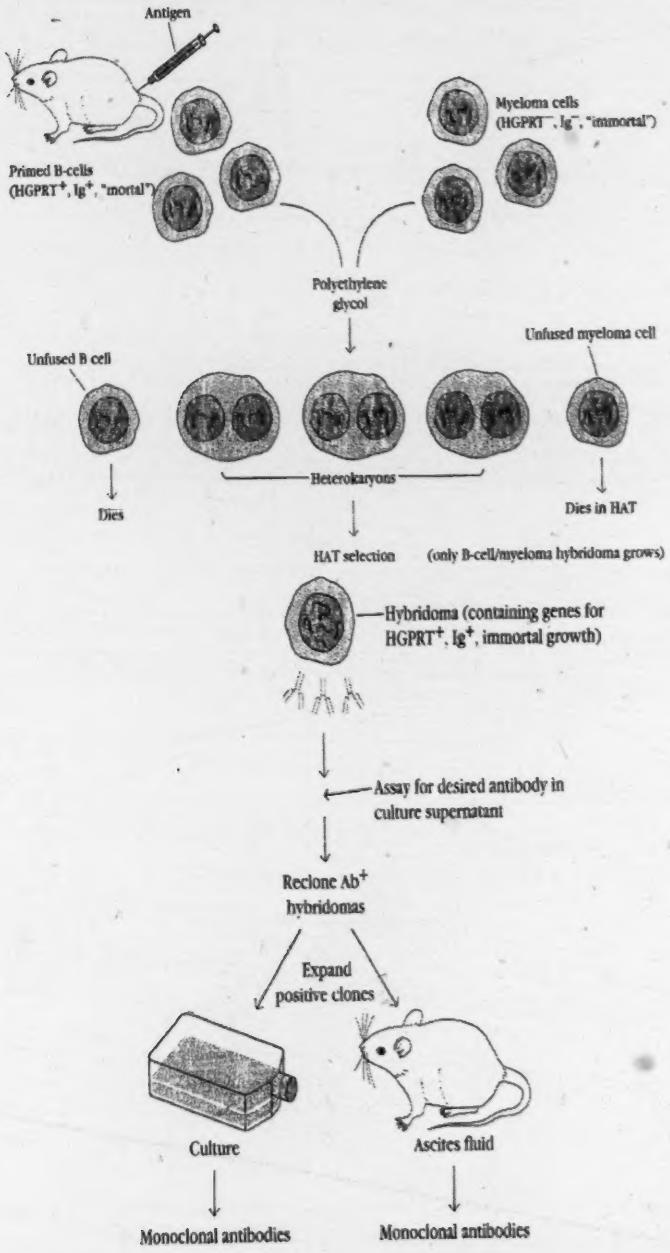

Figure 1.1: Hybridoma technique (Source: J.Kuby2000)

(thus, a monoclonal antibody), which could be purified and put to practical use. To make a immortal cell line producing a single antibody, Milstein and Kohler fused a mouse B cell with a cancer cell of B-cell origin called a myeloma. The resulting hybrid cell line, or hybridoma, grew easily in culture, an ability derived from its cancer cell parent, and secreted the same antibody as its other parent, the B cell (figure 1.1). Applied on an industrial scale, this was the beginning of Mabs for both diagnostic and therapeutic purposes. Thus the whole basis of hybridoma technology is to combine the growth characteristics of tumor cells with the antibody- producing capabilities of particular B cells. Antibodies produced by hybridoma clones are called monoclonal antibodies and are hence generally pure and homogeneous with respect to its antigen binding properties.

1.4 Human Monoclonal Antibodies

The production of human monoclonal antibodies has been hampered by a number of technical difficulties. The most important of these has been the lack of appropriate human myeloma cells that exhibit immortal growth, are susceptible to HAT selection, do not secrete endogenous antibody, and support continuous antibody production in the hybridoma made with them (Goding, J.W.1996). As an alternative to conferring immortality on human B cells by fusion to myeloma cells, normal human B-lymphocytes can be transformed with Epstein-Barr Virus (EBV) (Hahn, W.C et al., 1999). When B-lymphocytes are cultured with antigen in the presence of EBV, some of them acquire the immortal- growth properties of a transformed cell while continuing to secrete antibody. Cloning of such primed, transformed cells has permitted production of human monoclonal antibody, but the amounts secreted are generally small (Borrebaeck, C.A 1999). Another problem is the ethical issues of readily obtaining antigen-activated B

cells from the human lymphoid tissue equivalent to the mouse spleen. Generally, human hybridomas are prepared from human peripheral blood, which contains few activated B cells engaged in an immune response. It is possible to obtain B cells that have been activated by antigens present in vaccines that are normally given to people, but human volunteers cannot be immunized with the range of antigens that can be given to mice or other animals. To overcome this difficulty, cultured human cells are sometimes primed with antigen *in-vitro*. However, the *in-vitro* system cannot mimic the normal microenvironment of lymphoid tissue, as a result, the B cells usually produce only low-affinity antibodies (Hahn, W.C.1999). One way to avoid the need for *in-vitro* priming of human B cells is to transplant the cells necessary to support a human immune response from humans to SCID mice. Although normal mice would reject such transplants, they are readily performed in SCID mice, because these animals lack a functional immune system. Consequently, one can make SCID-human mice that contain human B and T cells. Following immunization of these mice, activated human B cells can be isolated from the mouse spleen and used to produce human monoclonal antibodies (Kipriyanov, SM. et al., 1999, Fishwild D.M 1996).

1.5 Advantages of Monoclonal antibodies

The main advantages of producing the monoclonal antibodies using immortalized hybridoma cell lines are- a) pure preparations of antibodies with known specificities. b) standardized antibody preparations whose properties are the same from batch to batch. c) relatively impure antigens can be used to raise specific antibodies. Monoclonal antibodies (Mabs) produced by fusion of a specific splenic B-cell to an

immortal B-cell tumor are able to make very fine structural or epitope distinctions in contrast to animal sera containing a spectrum of antibodies.

1.6 Types of Mabs

Developments in hybridoma technology have created four different types of Mabs.

- Murine (mouse) Mabs have been the primary focus of Mab generation to date. However, they produce variable results. Since mouse-produced antibodies are not identical to human antibodies, they are eventually recognized as foreign proteins by the human body and cleared from circulation by human antimouse antibodies (HAMA) (Schroff, R.W. et al., 1985, ShawlerD.L et al., 1985). These reactions are not a serious problem with Mab-based diagnostic and imaging products, where only a single application may be required. However, they are a major obstacle to the therapeutic use of murine antibodies. Most patients produce a HAMA reaction, which significantly reduces therapeutic efficacy and increases toxicity.
- Human Mabs do not produce a HAMA reaction, so they tend to succeed therapeutically and are less likely to produce allergic reactions. Unfortunately, it is extremely difficult to fuse human B-lymphocytes with myeloma cells (Borrebaeck et al., 1999). Several biotechnology companies are investigating novel ways to produce human Mabs, but this process appears to be much more expensive than murine-based systems.
- Chimeric Mabs use recombinant engineering technology and involve the assembly of diverse gene segments not normally found together in nature (Boulianne,G.L.et al.,1984).With this approach, recombinant genes are constructed that code for the

production of specific proteins (Mabs), in which selected segments from the mouse antibody are fused to complementary segments from the human antibody (Morrison S.L et al.,1984). While the chimeric antibody produced retains its binding specificity, it more closely resembles a natural human antibody. Therefore, it is less likely to produce a HAMA reaction (Riechmann, L et al., 1988).

- Humanized Mabs incorporate only the genes for the specific binding sites from the mouse antibody. Except for these "grafted" murine-binding sites, the rest of the antibody retains a distinctly human framework (Jones, PT. et al., 1986). Humanized Mabs represent a significant advance over initial chimeric technology. Several companies are involved in clinical testing of Mab-based therapies using these antibodies.

1.7 Bispecific Mabs

While humanized Mabs are engineered to mimic human antibodies as much as possible, bispecific antibodies (bsMabs) are engineered to give antibodies entirely new binding possibilities(Cao & Suresh 1998). Bispecific antibodies combine antigen-binding sites against two different predetermined antigens and have broad potential in therapy and diagnosis. A bsMab is a monoclonal antibody with two distinct binding specificities (Drakeman et al., 1997). In the field of cancer immunotherapy, bsMabs are developed to cross-link cancer cells and cytotoxic cells such as T cells, natural killers or macrophages. This requires the trigger arm's target to initiate cytotoxicity when bound. For example, bispecific antibodies have been used to specifically recruit a variety of different effector mechanisms; including cell-mediated cytotoxicity (by targeting CTLs, NK cells, phagocytes, etc.

1.8 Development of Antibody-based therapies

In the 19th century, it was discovered that the immune sera were useful in treating infectious diseases. Serum therapy was largely abandoned in the 1940's because of the toxicity associated with the administration of heterologous sera and the introduction of the effective antimicrobial chemotherapy (Rackemann FM. 1942). Recent advances in the technology of Mab production provide the means to generate human antibody reagents and reintroduce antibody therapies, while avoiding the toxicities associated with serum therapy (Casadevall A, et al., 1995). The disadvantages include high cost, limited usefulness against mixed infections, and the need for early and precise microbiological diagnosis. The potential of antibodies as anti-infective agents has not been fully tapped. Antibody-based therapies constitute a potentially useful option against newly emergent pathogens. In the mid-1990's successful implementation of anti infective therapy has become increasingly difficult because of widespread antimicrobial resistance, the emergence of new pathogens, and the occurrence of many infections in immunocompromised patients in whom antimicrobial drugs are less effective (Berkelman RL et al.,1995). Antibodies inhibit antimicrobial function through a variety of mechanisms, including inhibition of microbial attachment, agglutination, viral neutralization, toxin neutralization, antibody-directed cellular cytotoxicity, complement activation and opsonization (Roilides E, et al., 1992,Heinzel FP, 1995). The versatility of antibody-based therapies is illustrated by the ability of digoxin-binding antibodies to reverse digoxin toxicity (Smith T.W et al., 1982).

1.9 Polyclonal Sera Versus Monoclonal antibodies for Therapy

Most antigens possess multiple epitopes and therefore induce proliferation and differentiation of a variety of B-cell clones, each derived from a B cell that recognizes a particular epitope. The resulting serum antibodies are heterogeneous, comprising a mixture of antibodies, each specific for one epitope. Such a polyclonal antibody response facilitates the localization, phagocytosis, and complement-mediated lysis of antigen; it thus has clear advantages for the organism *in-vivo*. Unfortunately, the antibody heterogeneity that increases immune protection *in-vivo* often reduces the efficacy of an antiserum for various *in-vitro* applications. For most research, diagnostic and therapeutic purposes monoclonal antibodies derived from a single clone and thus specific for a single epitope, are preferable (J.Kuby 2000). Immunized animals may produce several thousand clonotypes against a given epitope (J.Kuby 2000) and it is therefore, impossible to obtain antisera with similar properties from different animals. Even antisera taken successively at different times from the same animal differ in their properties. Monoclonal antibodies, on the other hand, are produced by a single clone, which can be maintained *in-vitro* over very long periods and can be used to generate large quantities of identical antibodies. A uniform, specific and constant affinity for a certain epitope is thus obtained. The higher specific activity of Mabs may also translate into greater therapeutic efficacy. Mab formulations are superior to polyclonal sera in homogeneity, constancy, specific activity, and possibly safety. For some infections, polyclonal preparations may be superior to Mab because polyclonals contain antibodies to multiple epitopes (Arturo Casadevall 1996).

1.10 Applications of Mabs

Monoclonal antibodies are now established diagnostic, imaging, and therapeutic biologicals in clinical medicine. Initially, monoclonal antibodies were used primarily as *in-vitro* diagnostic reagents. Among the many monoclonal antibody diagnostic reagents now available are products for detecting pregnancy, diagnosing numerous pathogenic microorganisms, measuring the blood levels of various drugs, matching the histocompatibility antigens, and detecting antigens shed by certain tumors (Bolhius 1992).

Mabs act as specific probes that are directed at the protein that induced their formation, they can be used successfully in clinical applications. For instance, Mabs can direct immune system activity by seeking out target cells and attracting immune cells (such as monocytes, macrophages and lymphocytes) to the targeted cell. These immune cells then kill the targeted cell through an immune system process known as antibody-dependent cell-mediated toxicity (Drakeman D et al.,1997). Mabs can also induce an immune process called complement-mediated cytotoxicity, in which Mabs along with complement lyse a target cell. Complement sets off a biochemical reaction called the complement cascade that results in cell destruction by attracting immune cells to a targeted area for cell-mediated toxicity (Kuby J 2000). Additionally, Mabs can be directed at target molecules needed for cellular growth or differentiation. For example, approximately one third of all patients with breast cancer carry a specific protein Her 2 neu on the surface of their tumor cells (Drakeman D.L et al.,1997). When a Mab directed at this protein, is used in combination with traditional chemotherapy, patients experience a greater degree and duration of therapeutic response. This results in an increased rate of

overall survival when compared to treatment with chemotherapy alone (Mukherjee.J et al.,1994).

Conjugated Mabs are monoclonal antibodies that are combined with radioisotopes, toxins or drugs for targeted delivery to specific cells. This enables the physician to deliver a greater concentration of the drug to the diseased area of the body, thus reducing the toxicity associated with therapy by avoiding exposure of healthy tissue to the agent. For example, a Mab conjugated with the chemotherapeutic agent doxorubicin in liposome is 10 times more potent than doxorubicin administered alone (Stuart D et al.,1997).

Some Mabs are conjugated with drug carrying liposomes to reduce the ability of these drugs to be taken up in certain critical organs, such as the heart, kidneys or gastrointestinal tract. This approach reduces drug toxicity. As one example, a Mab that is conjugated with the chemotherapeutic agent doxorubicin (which is also cardiotoxic) delivers this drug to breast cancer patients without causing damage to heart tissue (Fonseca et al.,1995).

A new approach to Mab therapy is the development of bispecific Mabs that are engineered to be directed to both a tumor cell and an immune system cell. The Mab can then bind the cells together, resulting in improved cell-mediated toxicity. Current trials using this approach are underway in patients with Hodgkin's lymphoma, where it appears to be a promising therapy (Drakeman D.L et al.,1997).

Finally, Mab fragments and recombinant immunotoxins can be directed toward site-specific antigens, where their small size enables them to gain greater access to tumors and other cells. Additionally, they are more rapidly cleared from the body than

larger Mabs. This approach is being investigated for the treatment of digitalis toxicity, where Mab fragments bind to circulating digoxin and aid in clearing it from the bloodstream (Smith T.W et al.,1982). Mabs can also be conjugated with a radioisotope, and hence they are well suited for use in diagnosing and monitoring disease (Chodak, G.W 1993). This was one of the earliest uses for biotechnology. The first products using Mabs to diagnose disease were approved by the FDA in 1981, and Mabs have been used in diagnostic imaging since 1992. For example, in cancer diagnostics, Mabs target specific antigens found on cancer cells, such as carcinoembryonic antigen, are conjugated with a radioisotope. These Mabs are then administered to patients, where they target tumor tissue when evaluated with computer tomography.

Another novel application is the use of Mabs as Abzymes (catalytic antibodies), to catalyze the chemical reactions. Like enzymes, antibodies of appropriate specificity can stabilize the transition state of a bound substrate, thus reducing the activation energy for chemical modification of the substrate (J Kuby 2000).

1.11 Treatment with Mabs

Several Mabs are now available commercially for the treatment of a variety of diseases. More than a hundred are in various phases of clinical trials. Key diseases and conditions where Mab therapy is used include:

- Non-Hodgkin's lymphoma: rituximab is a chimeric Mab that targets antigens on B-lymphomas and induces complement-mediated and antibody-mediated cellular toxicity (Davis,T.A et al.,1999). I-131 tositumomab works like rituximab against

B-lymphomas, but is conjugated with a radioisotope delivered directly to the tumor (Vose, J.M et al.,1999).

- Breast cancer: trastuzumab is a murine Mab that targets HER2 protein found in one third of women with breast cancer and inhibits tumor growth. This Mab is associated with cardiotoxicity, which is intensified if used with cardiotoxic chemotherapeutic agents, such as doxorubicin or cyclophosphamide (Goldenberg, M.M 1999).

- Cardiovascular disease: abciximab targets clotting proteins in order to prevent thrombus formation in patients undergoing percutaneous transluminal coronary angioplasty (Juberlirer, S.J 1999).

- Crohn's disease: infliximab is a chimeric Mab that binds to TNF-a (tumor necrosis factor) to reduce the intestinal inflammation this protein causes (Maini,R et al.,1999).

- Rheumatoid arthritis: etanercept is a Mab fragment linked to the TNF receptor to block TNF from binding to tissue to prevent inflammation (Elliott, M.J et al., 1994).

- Organ transplantation: muromonab-CD3, daclizumab and basilixmab are used in the prevention and treatment of organ rejection (Martin J.G et al., 2000).

    - Muromonab is a murine Mab associated with significant side effects and HAMA reactions.

- Daclizumab and basilixmab are humanized Mabs associated with fewer toxicities.

Respiratory tract disease: palivizumab is a human Mab used for the prophylaxis of serious lower respiratory tract disease, caused by respiratory syncytial virus (RSV), in pediatric patients at high risk for RSV disease (Saez-Llorens,X et al.,1998).

1.12 The biosynthesis of immunoglobulins

Cellular basis: Cellular immunologists have convincingly demonstrated that cells of the lymphoid system are responsible for immune reactions such as delayed hypersensitivity, graft rejection and antibody formation. The principal cells involved are the lymphocytes. These cells, although morphologically indistinguishable, can be classified by functional differences into two categories, one category which requires the thymus gland for development- the so-called thymus-dependant lymphocytes or T lymphocytes-and a second which develops independently of the thymus- the B-lymphocytes. However, all the lymphoid stem cells originate from the bone marrow. The T-lymphocytes are responsible for the reactions of cell mediated immunity and the B-lymphocytes are precursors of cells, which synthesize and secrete antibody- the plasma cells. While T-lymphocytes do not themselves produce antibody, they do co-operate with B-lymphocytes in antibody production, which has led to their description as 'helper' cells in this context. A third cell type- the macrophage-has also been implicated in the immune response. The major role of macrophages appears to be that of antigen processing. T and B-lymphocytes can be distinguished on the basis of differences in surface antigens. The clonal selection theory of Burnet F.M (1957) is widely accepted as a working hypothesis

for antibody synthesis. This theory suggests that individual lymphocytes have the genetic capacity to make one, or possibly a small number of particular antibody specificities. The lymphocytes have, on their surface, immunoglobulin or immunoglobulin like receptors. These receptors have the same specificity for antigen as the antibody which the cell can make when differentiated. Thus, when an antigen is presented to the lymphoid cells of an animal, cells with receptors which can react with the antigen will be stimulated to differentiate and will ultimately produce a clone of cells producing antibody with specificity towards the inducing antigen. Not all the cells differentiate into antibody forming B cells (plasma cells), some become long –lived antigen-reactive 'memory cells'. It is now clear that both B and T-lymphocytes can bind antigen via surface receptors. The receptors of B-lymphocytes are immunoglobulins. Each cell has approximately 10^4-10^5 immunoglobulin molecules on its surface. It is thought that the immunoglobulin receptors are oriented with the Fc regions towards the B-lymphocyte surface leaving the antibody binding sites free to react with the antigen (Freshney I 2000).

Synthesis, assembly and secretion: Once antigen-induced selection, differentiation and proliferation of antibody forming B-lymphocytes have occurred, antibody is produced and appears in the circulation. Immunoglobulin synthesis is achieved by plasma cells by the normal processes of protein synthesis. Heavy and light immunoglobulin polypeptide chains are synthesised on separate ribosomes. Heavy chains are synthesised on polyribosomes consisting of 11-18 ribosomes. Heavy and light chain synthesis is normally balanced to result in the formation, assembly and secretion of completed molecules. Completed immunoglobulin molecules are held in their stable configuration

by covalent and non-covalent bonds. Inter- and intra- chain disulfide bonds play an important part in the stabilization. An attractive model for the secretion of immunoglobulin has been suggested by Swenson et al., 1967 and is essentially as follows: Immunoglobulin chains are synthesised and partially assembled on polyribosomes. Carbohydrate is added to the molecule in the rough endoplasmic reticulum. The chains enter the cisterne of the endoplasmic reticulum and the four-chain molecule is formed in the cisternal space. The completed molecule is transported to the golgi complex where further carbohydrate is added. After this, it is presumed that the molecule is contained in secretory vesicles prior to secretion.

1.13 Antibody Structure and Function

Immunoglobulins, or antibodies, are complex, heterogeneous mixtures of proteins that exhibit two fundamental types of structural variation. Subtle structural differences in their antigen combining sites, or variable regions, account for their unique antigen binding specificities. Structural differences outside their antigen combining sites, in the so called constant regions, correlate with the different effector functions mediated by antibodies, such as complement activation or binding to one or more of the antibody Fc receptors expressed on monocytes and granulocytes (Alzari et al., 1988).

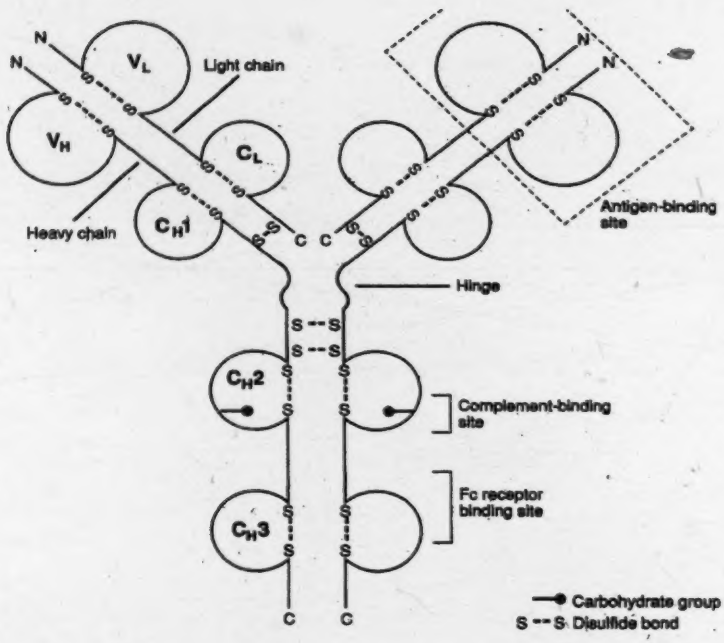

Figure 1.2 Antibody structure (Source:Andrew H 1991)

An antibody (Ab) is a glycoprotein directed against antigen (Ag). An antigen is a substance that can elicit an immune response. Antibodies are composed of two types of polypeptide chains (figure 1.2): light chains (about 25 Kd each) and heavy chains (range from 50 to 77 Kd). Both heavy and light chains have N-terminal domains or regions, where somatic mutations most likely occur and the resulting protein sequence is highly variable (also the site of Ag binding). Both heavy and light chains have regions or domains where the protein sequence is highly constant. The constant region of the heavy chain of the Ab determines the class of antibodies. There are 5 classes (IgG, IgM, IgA, IgD, and IgE) and some classes are even further defined into subclass. Heavy chains of all classes associate with light (L) chains of two isotypes κ and λ.

1.14 Animal cell culture media

Explant tissue and the resulting cell lines need to be maintained in an environment that provides the optimum conditions for growth and survival. The cell culture medium is probably the most important single factor in promoting cell survival and proliferation. The medium must provide all the essential nutrition, buffering and gas exchange the tissue needs.

There are many different media formulations that have been developed to meet the requirements of particular cell types or to achieve particular objectives. Immediate survival media simply needs to provide a source of energy and to maintain the correct osmolarity. These basic requirements may be met by a combination of inorganic salts and glucose. Such conditions can be achieved by a defined medium consisting of a balanced salt solution containing glucose. In contrast, culture media for long term cultivation of cells needs to be more sophisticated with a variety of factors. These might include a variety of amino acids, vitamins, growth factors and hormones. These can be supplemented in the media as pure chemical added in controlled amounts, so called as defined media or achieved by adding serum to the media so called as the undefined media. Media not only need to supply the chemicals needed for growth but they must also provide the right physical conditions for cells. However the surface tension and viscosity are also important considerations. Surface tension in the medium can be used to promote adherence of cells to the substratum. Balanced salt solutions are called synthetic media as they contain a variety of mineral salts. There are certain types of media that don't support the growth of the cells without the supplementation of the serum such media is called as

minimum essential medium (MEM). Usually the serum is derived from the fetal bovine, bovine, fetal horse, horse, chicken, goat, human, porcine, rabbit and sheep.

1.14.1 *Nutritional components of media*

Amino acids: Essential amino acids are those which cannot be synthesized from raw materials by heterotrophic organisms. These amino acids, together with cysteine and tyrosine must be incorporated into a chemically defined synthetic medium even if a serum supplement is to be used to grow the cells. Individual requirement may vary and non-essential amino acids may need to be added to compensate for particular cells' inability to synthesize adequate amounts of them or because they become leached out into the medium. The concentration of amino acids influences cell yield and also affects survival and growth rates. Amino acid deficiency inhibits cell division, induces chromosomal damage and increases lysosomal activity and cell death. Imbalances of amino acid concentrations may also produce karyotypic changes. The most rapid use of amino acids occurs during the lag phase of the growth cycle, and cystine, glutamine, isoleucine and serine are used up very quickly. Glutamine is used by most cells and provides an essential source of carbon and energy. It is the most unstable (labile) of the amino acids and needs to be replenished by regular medium changes. It is also likely that glutamine has a role in the formation of molecules involved in cell adhesion.

Nucleic acid precursors: Adenosine, guanosine, cytidine, uridine and thymidine are often added to media, particularly if folic acid is in short supply such as in low density cultures.

Carbon source: The growth of cells depends on the availabilty of carbon sources, usually glucose and glutamine. Both nutrients provide unique biosynthetic precursors. Glucose is

required for nucleoside synthesis (Wice et al.,1981; Renner et al.,1972), as well as for the synthesis of glucosamine 6-phosphate and the precursors glyceraldehyde 3-phosphate (Mc Keehan 1986). It is metabolized principally by glycolysis to form pyruvate, which may be converted to lactate or acetoacetate and may enter the citric acid cycle to form CO_2. The accumulation of lactic acid in the medium, particularly evident in transformed cells, implies that the citric acid cycle may not function entirely as it does *in-vivo*. Glutamine is required for purine synthesis and for the formation of guanine nucleotides (Raivio and Seegmiller 1973). It is also the primary amino group donor in the synthesis of pyrimidines, amino sugars, and asparagine. Although some cell lines can use glutamate directly (Griffiths and Pirt 1967), most cells require glutamine for optimal growth.The recent data have shown that much of its carbon is derived from glutamine rather than glucose. This finding may explain the exceptionally high requirement of some cultured cells for glutamine or glutamate.

Vitamins: Vitamins are required to act as cofactors in metabolism.

Growth factors: The family of polypeptides that are mitogenic *in-vitro* are called growth factors. Growth factors and cytokines act as paracrine factors, growth factors may act synergistically or additively with each other or with other hormones and paracrine factors. Some of the functions carried over by the growth factors in tissue culture are promotion of mitosis, induction of anchorage-independent growth and loss of contact inhibition. Natural clot serum stimulates cell proliferation more than serum from which the cells have been removed physically (e.g.,by centrifugation).This increased stimulation appears to be due to the release of platelet-derived growth factor (PDGF) from the platelets during clotting. PDGF is one of a family of polypeptides with mitogenic activity

and is probably the major growth factor in serum. Most growth factors are available in minute amounts in serum, usually in the order of nanograms or picograms per ml. Some, like the colony stimulating factors, act specifically on cells at a distinct stage of differentiaion.

Hormones: Insulin is essential for the growth of nearly all cells in culture. It is very sensitive to inactivation by cysteine and has a very short half-life so large amounts need to be added to the culture medium.

Attachment and spreading factors: Most non-transformed cells have to attach themselves to a solid substrate in order to grow. Only haemopoetic and transformed cells can multiply without attachment.

Binding proteins: Factors which are of low molecular weight are often carried into cells by binding onto transport proteins. Examples of transport proteins are, albumin which carries vitamins, lipids and hormones into cells and transferrin which is involved in the binding and transport of iron.

Fatty acids: Cells require varying amounts of essential fatty acids, phospholipids and cholesterol. Prostaglandins E and $F_{2\alpha}$ are involved in cell growth, possibly acting in conjunction with EGF and other growth factors.

Trace element: Copper, zinc, cobalt, manganese, molybdenum and selenium are present in serum and are thought to be involved in activating enzymes and protecting against free radicals which cause damage to DNA.

Serum: Serum is a very complex mixture of many small and large molecules with growth promoting and growth stimulating activities. Serum is used in cell culture medium to provide:

- Hormonal factors which promote cell growth and cellular functions
- Growth factors which promote cell growth and cellular functions
- Attachment and spreading factors
- It has been found that some antibodies produced in serum free medium exhibit a simpler glycosylation pattern and altered pharmacokinetics compared with antibodies produced in serum containing media (Maiorella et al., 1990).

Serum supplemented cell culture media typically provides higher productivity of the cell line. The presence of serum is therefore beneficial for cell line productivity and stability as well as product quality.

Serum provides an enormous variety of the necessary ingredients to successfully cultivate animal cells. It is not surprising that it finds extensive use as a supplement in a wide variety of media. However, its use does present many potential **disadvantages**. They are:

- Sera from different animals differ widely in their properties. Even batches of sera from the same species vary enormously, so all serum supplements have to be batch tested extensively from time to time. This procedure is time consuming and expensive;
- Under normal physiological conditions, most cells do not come into contact with serum except during wound healing, so it is not the most natural medium for culturing cells and this can complicate the process of extrapolating *in-vitro* experimental results to the *in-vivo* situation;

- Some components of serum are actually cytotoxic under some conditions. Serum often contains selective inhibitors, bacterial endotoxins and lipids, and polyamine oxidase which reacts with polyamines produced by proliferating cells to form toxic polyaminoaldehydes which can suppress cell yields;
- Specific growth factors may be present in inadequate amounts to grow some cells and purified growth factors may still have to be added;
- Fetal calf serum, which is used for many of the more demanding cell types is very expensive and limited in supply. It needs to be used at concentrations of between 5-20% depending on the cell type and this can make it prohibitively expensive. Hormone levels may vary and foetal calf serum often contains very high levels of the enzyme arginase, which depletes the culture of the essential amino acid arginine. Sera from newborn and adult animals are used but often the latter have high levels of gammaglobulins(antibodies) which may cause subsequent problems.

1.14.2 Serum substitute-Serum Free Medium

Low serum and serum-free medium are particularly valuable where it is vital to control the culture conditions precisely. A problem with most serum –free media is that they are often highly specific to one or two cell types, so a different formulation for different types of cell lines is required. Some of the advantages are:

- Risk of introducing contaminants(bacteria, viruses, mycoplasma) is reduced;
- Serum cytotoxicity and protein interference is reduced;
- Serum free media can be less expensive;
- Culture products are easier to purify.

Disadvantages of Serum Free Media

Serum-free media are not without disadvantages:

- Multiplicity of Media: Each cell type appears to require a different recipe, and cultures from malignant tumors may vary in requirements from tumor to tumor, even within one class of tumors.
- Selectivity: Unfortunately, the transition to serum-free conditions, however desirable, is not as straightforward as it seems. Some media may select a sub-lineage that is not typical of the whole population, and even in continuous cell lines, some degree of selection may still be required. Cells at different stages of development may require different formulations, particularly in the growth factor and cytokine components.
- Cell Proliferation: Growth is often slower in serum-free media, and fewer generations are achieved with finite cell lines.
- Availability: Although improving steadily, the availability of properly controlled SFM is quite limited, and the products are often more expensive than conventional media.

1.14.3 Non-nutritional medium supplements

pH: Most cell lines require pH of 7.4. Hybridoma and some tumor cells can survive at a pH as low as 6.8 but growth is impaired. The pH of the culture is influenced by the buffering components of the medium, the amount of headspace (in a closed system), and the concentration of glucose. Medium is usually buffered using a CO_2-bicarbonate system which is analogous to the physiological system. Phosphates in the medium also aid buffering for some cell lines. The headspace in a closed, batch culture vessel should contain air supplemented with 5%(v/v) CO_2. Gas exchange occurs at the medium-

air interface, and is further facilitated by the continuous stirring of the medium. However, as the cells grow and generate CO_2 there comes a point when gas exchange can no longer occur and the pH of the medium becomes acidic. All powdered media are produced without $NaHCO_3$ to increase the stability.

Oxygen: Cells chiefly rely upon the dissolved O_2. Providing the correct O_2 tension is , therefore, always an important strategy. Most dispersed cell cultures prefer lower oxygen tensions, and some systems do better in less than the normal level of atmospheric oxygen tension. Oxygen is very insoluble in medium (approximately 7.6μg /mL) and cells typically use it at a rate of 5-7μg/min per 10^6 cells. Thus a culture of $2x10^6$cells/mL would very rapidly run out of oxygen. Clearly it is necessary to supply oxygen throughout the culture period. Surface aeration, sparging, medium perfusion or increasing the partial pressure of O_2 in the headspace can all help to maintain adequate oxygen levels in the system. A drastic change in the dissolved oxygen concentration may provoke a situation, where anaerobic stress proteins are produced at the expense of normal protein synthesis.

CO_2 and Bicarbonate: Carbon dioxide in the gas phase appears in the medium as dissolved CO_2 in equilibrium with HCO_3- and lowers the pH. Because dissolved CO_2, HCO_3^- and pH are all interrelated, it is difficult to determine the major direct effect of CO_2. The atmospheric CO_2 tension will regulate the concentration of dissolved CO_2 directly, as a function of temperature (Jayme et al.,1985).

Surface tension and Foaming: The effects of foaming have not been clearly defined, but the rate of protein denaturation may increase, as may the risk of contamination. Pluronic

F68 0.01-0.1% helps prevent foaming in this situation by reducing surface tension and may also protect cells against shear stress from bubbles.

*Antibiotics:*Antibiotics were originally introduced into culture media to reduce the frequency of contamination. However, the use of laminar-flow hoods, coupled with strict aseptic technique, makes antibiotics unnecessary. Indeed, antibiotics have a number of significant disadvantages:

1. They encourage the development of antibiotic-resistant organisms.
2. They hide the presence of low-level, cryptic contaminants that can become fully operative if the antibiotics are removed, the culture conditions change, or resistant strains develop.
3. They may hide mycoplasma infections.
4. They have anti-metabolic effects that can cross-react with mammalian cells.
5. They encourage poor aseptic technique.

*Temperature:*Cultured cells will tolerate considerable drops in temperature, can survive several days at 4°C, and can be frozen and cooled to –196°C , but they cannot tolerate more than about 2°C above normal (39.5°C) for more than a few hours and will die quite rapidly at 40°C and over. A temperature shock above the optimum for a time period of 1-2 hours results in the production of heat shock proteins (HSPs) at the expense of normal protein synthesis (Piper 1987).

1.15 Perfluorocarbons (PFCs)

The PFCs are low molecular weight (450-500 Da) linear or cyclic hydrocarbons, occasionally containing oxygen or nitrogen atoms, and in which the hydrogen atoms of the carbon chain have been replaced by fluorine that can dissolve large volumes of respiratory gases.

Artificial oxygen (O_2) carriers aim at improving O_2 transport and O_2 unloading to the tissue. Artificial O_2 carriers may thus be used as an alternative to allogenic blood transfusions or to improve tissue oxygenation and function of organs with marginal O_2 supply. Such substances have also been named as 'artificial blood'. This term, however, is a misnomer since these substances are designed to exclusively carry O_2 and CO_2 while they are devoid of other properties of blood such as coagulation and anti infectious properties (T.F. Zuck et al.,1994, Donat R S 2000). Currently evaluated artificial O_2 carriers can be grouped into modified hemoglobin solutions and fluorocarbon emulsions.

The first generation PFC emulsion-based blood substitute developed was Fluosol(Green Crosss, Japan), which contained 14.0% (w/V) of perfluorodecalin ($C_{10}F_{18}$), and 6.0% (w/v) of perfluorotripropylamin [$(C_3F_7)_3N$].

The main objectives of the research and development effort to produce superior PFC emulsions to supersede Fluosol and other first generation emulsions were (I) identification of PFCs with biocompatibility and excretion properties suitable for *in-vivo* use, (II) improvement in stability characteristics, through the use of perfluorinated stabilisers and non-poloxamer surfactants and (III) the development of sterilisable emulsions having significantly increased PFC content conferring superior oxygen-carrying capacity. The two PFCs most widely studied as core constituents of injectable

emulsions are the linear molecule, perflubron(perfluoro-octyl bromide ;$C_8F_{17}Br$ and the bicyclic compound , perfluorodecalin.The molecular weights of both compounds fall within the range 460-500,which is recognised as that giving acceptable tissue retention times (Lowe,K.C 1994; Zuck, T.F et al 1994; Spence,R.K et al 1996; Riess, J.G et al 1997). It has been emphasized (Riess 1984) that the excretion rate of PFCs from the body depends primarily on molecular weight, with molecular structure and the presence of cycles or heteroatoms having minimal influence.

1.15.1 ***Chemistry of PFCs****:* Perfluorocarbons are compounds consisting in a narrow sense of carbon and fluorine atoms only; in a broader sense all compounds are summed up under this term having all their hydrogen atoms replaced by flourine, and containing single bonds only, and fluorine is bond to carbon only. By special methods, these compounds are perfluorinated, i.e. all hydrogen atoms are replaced by fluorine, and all multiple bonds are saturated. As a consequece of the high ionization potential of fluorine and especially of its low polarizability, the intermolecular interactions in liquid perfluorocarbons are very weak, and the surface energies are low. Due to its extraordinary high electronegativity, fluorine is always electron withdrawing when bonded to carbon, causing a relatively high ionic character of the C-F bond making it stronger than any other C-X bond. Because of the compartively small size of fluorine, all hydrogen atoms in an organic molecule can be replaced principally by fluorine with the molecular structure remaining essentially intact. Due to the slightly larger fluorine atoms, the resulting perfluorocarbons are somewhat stiffer than that of the hydrogenated.Their carbon skeleton and carbon-carbon bonds are completely shielded by fluorine, making

them less accessible to any chemical attack (Rudiger et al.,2000). In summary, PFCs are of extraordinary chemical as well as thermal resistance. The fluorine and C-F bond peculiarities imply many specific properties of perfluorocarbons, several of them are valuable from a medical point of view (Rudiger et al.,2000).

1.15.2 ***Properties of Perfluorocarbons:*** Perfluorocarbons are hydrocarbons in which most or all of the hydrogen atoms have been replaced with fluorine. This progressive substitution of fluorine for hydrogen leads to an increase in molecular mass, and so PFC liquids are much heavier than hydrocarbon oils such as mineral oil. These may be either aromatic or aliphatic compounds. These must not be confused with volatile chlorofluorocarbons (CFCs) which release highly reactive chlorine atoms into the stratosphere, thus damaging the earth's ozone layer. Gas solubility in PFCs increases linearly with partial pressure, approximating Henry's Law.

They have very low surface tension allowing them to wet any solid surface; Are strongly hydrophobic but also oleophobic, consequently, they are immiscible with water, and very limited miscible with oleophilic liquids; the specific densities of PFC's are near 2g/cc.

PFCs are petroleum-based compounds synthesized by substituting fluorine for the hydrogen atoms of hydrocarbons. Lack of strong interactions between PFC molecules enables gaseous oxygen to enter the spaces between them. Oxygen is dissolved physically only, there are no specific interactions with the PFC (Mack HG et al., 1987). PFC dissolves about 20 times more oxygen than water does and even more carbon dioxide; Oxygen solubilities higher than 10 to 20 times that of pure water are found in several commercially available PFCs (Fluorinet 1987). On the contrary, due to the very

weak intermolecular interactions in PFCs, there is sufficient free space between the PFC molecules to be occupied by oxygen or other low molecular gases.

In summary, fluorocarbons are characterized by exceptional chemical and biological inertness, extreme hydrophobicity, lipophobicity, high gas –dissolving capacities, low surface tensions, high fluidity and high density, absence of protons, and magnetic susceptibilities comparable to that of water. These unique properties are the foundation for a range of biomedical applications (Table 1.1)

Table 1.1:Examples of Biomedical Application for PFC Liquids and their Emulsions.

Product	Company	Year	Status and Purpose
Fluosol DA	Green Cross Corp. Japan	1990	Approval of emulsion for clinical use in coronary balloon angioplasty
Perftoran	Russia	1996	Approval for haemorrhagic shock Pateints. Perfusion of isolated human Organs
Oxygent	Alliance Pharm. Corp. USA	1997	Phase II-temporary tissue Oxygenation in 250 surgical patients
		1998	Clinical trials with more than 340 patients
		end of 1998	Phase III studies started
Liquivent	Alliance Pharm Corp. USA	actual	Liquid ventilation fluid under testing Phase III ongoing
FC-40	3M company USA	1992	Increase cell density and Mab synthesis as cell culture media supplements
FMDC	3M company USA	1992	Increase cell density and Mab synthesis as cell culture media supplements

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1.15.3 ***Oxygen Solubility, Transport and Delivery of PFCs***: PFCs are chemically inert, fluorine-substituted hydrocarbons that can dissolve large volumes of non-polar gases. A typical value for oxygen solubility in PFC liquids (STP) is 45mL /100 mL; the corresponding value for CO_2 can be over 200 mL /100mL. Gas solubility in PFC liquids

decreases in the order $CO_2>>O_2>CO>N_2$ which correlates with the decrease in molecular volume of the solute (Lowe K.C 1997). Linear PFCs, such as per fluoro–octyl bromide disssolve oxygen more efficiently than cyclic molecules such as perfluorodecalin. In general, oxygen solubility in PFC is inversely proportional to the molecular weight (MW) and directly related to the number of fluorine atoms present (Spence RK 1996). Oxygen solubility in PFC liquids is about 20-25 times greater than in either water or blood plasma under the same conditions. Unlike the chemical binding of O_2 to the porphyrin-iron sites of Hb, O_2 dissolution in PFCs is a simple passive process, in which gas molecules occupy so-called 'cavities' within the PFC liquids. In contrast to the characteristic sigmoid binding curve of O_2 to Hb, O_2 solubility in PFC's and their emulsions increases linearly with partial pressure essentially following Henry's Law (K.C Lowe 1999). Oxygen dissolves in PFC droplets as they pass through the lungs. The total amount of O_2 dissolved in a PFC emulsion depends on the concentration of PFC and the solubility coefficient of gas. The alveolar O_2 loading in PFC's is linearly related to the pO_2 and this can be significantly enhanced when the recepient breathes supplementary O_2. O_2 delivery by PFC is much simpler than the release of O_2 from Hb, where the gas has to cross the red cell membrane, pass through the plasma, and then diffuse through both the membranes of the endothelial cells and those of the tissues it is supplying. Oxygen delivery by PFC's appears to be more complex than simple "bulk transport: it has been suggested that PFCs may facilitate the transfer of oxygen into tissues by acting as "stepping stones" between red cells and blood vessel walls in vessels with rapid flow (e.g. arterioles) (Faithfull NS 1994). PFC emulsion droplets in the circulation are postulated to flow mainly in the plasma layer that forms close to the vessel walls as a

result of erythrocyte streaming (Keipert PE 1998). In the microcirculation, PFC droplets will occupy the plasma gaps between red blood cells and thereby perfuse even the smallest capillaries. Some perfusion by PFC's will be expected to occur in vessels that effectively exclude red cells, as a result of local vasoconstriction or ischaemia. Thus, under such conditions, a PFC emulsion will make a significant contribution to overall tissue O_2 delivery.

1.15.4 ***PFC emulsions:*** PFC liquids are immiscible with aqueous systems, including blood and other body fluids, and can be injected safely into the vascular system in an emulsified form. Emulsions of PFCs have been evaluated clinically as, for example, temporary respiratory gas-carrying fluids for intravascular administration. PFC emulsions can only replace the respiratory gas-carrying functions of whole blood. Because the PFCs are not hydrosoluble, their use as pure solution in the intravascular space is impossible, but they can be administered as emulsions, containing a dispersion of fine particles, suspended in an isotonic electrolyte solution. In order to produce emulsions, which are stable at room temperature, emulsifying agents (surfactants) are necessary. The properties of PFC emulsions depend on both the components of the emulsion, but also on the proportion of the various components and on the sizes of the emulsion particles, which influences the stability of the emulsion, the surface area available for gas exchange, the viscosity, and the intravascular half-life. The emulsions are prepared using ultrasonication or high-pressure homogenization, but sonication appears to be partially destructive, liberating free fluoride ions, altering the composition of the emulsion, and increasing the risk of toxicity (B Remy et al.,1999).

Emulsion stabilisation issues: A major objective in the production of second-generation, injectable emulsions was to improve stability and extend shelf life. Emulsions are thermodynamically unstable systems and, in PFC-based formulations, the principal mechanism by which droplets grow is through a process of molecular diffusion known as Ostwald Ripening. During this process, PFC molecules from smaller droplets diffuse through the continuous phase to the larger droplets which progressively increase in size at the expense of the former (Kabalnov,A et al.,1995). Ostwald ripening in emulsions of perfluorodecalin can be retarded by the addition of for example, a small amount of a perfluorinated, high molecular weight, high boiling point oil (HBPO) additive, such as perfuoroperhydrophenanthrene ($C_{16}F_{26}$) (Sharma,SK et al.,1989). In some studies, 2%(w/v)soya oil was also used (Johnson, O.L et al.,1990). Effective stabilisation of PFC emulsions against Ostwald ripening mediated aging has been a major hurdle for researchers to overcome in the development of room temperature-stable formulations for biomedical applications.

Biomedical applications for perfluorochemicals and their emulsions include their use as pump-priming fluids for cardiopulmonary bypass, lung ventilation fluids, anti-cancer agents, organ perfusates and cell culture media supplements(K.C Lowe 1999; Moo H. Cho et al.,1988).

1.15.5 ***Pharmacokinetics of PFCs:*** Emulsion droplets of PFCs are approximately a tenth of a micron (1/70 the size of a red blood cell), they may also be able to perfuse and oxygenate tissue more effectively than red cells. Unfortunately, because these droplets are seen as foreign bodies, they are quickly cleared from the circulation by the

reticuloendothelial system (with a plasma half-life of approximately 12 h) (Tremper KK et al., 1982). Once out of circulation, the PFC accumulates in the liver and spleen and then is slowly transported to the lung, where it leaves the body chemically unchanged in the expired gases, with a tissue half-life measured in months.

After intravenous application, the droplets of the emulsion are taken up by the reticulo-endothelial system (RES). This uptake into the RES determines the intravascular half-life (D.R Spahn et al., 1994, J.G.Riess et al 1992., P.E Keipert et al.,1998). At the present time no exact data are available on the intravascular half- life in humans. After the initial uptake of the fluorocarbon emulsion into the RES, the droplets are slowly broken down, and the fluorocarbon molecules are taken up in the blood again (bound to blood lipids) and transported to the lungs, where the unaltered fluorocarbon molecules are finally excreted by exhalation. At the present time, metabolism of fluorocarbon molecules is unknown in humans (D.R Spahn et al.,1998). The ability of fluorocarbon emulsion to transport and efficiently unload O_2 is undisputed.

1.15.6 ***The potential of highly fluorinated molecular materials in medicine and biology:***

Delivering oxygen to tissues becomes vital when a patient's hemoglobin level is declining. Oxygen delivery can then be achieved using a fluorocarbon emulsion (Proceedings of Vth Int Symp Blood Substitutes, San Diego, U.S.A 1993). Such emulsions are expected, when combined with autologous blood transfusion stratagies, to play a major role in surgery by increasing safety at low hematocrits and avoiding or reducing donor blood transfusions. Fluorocarbon-based oxygen carriers should also prove beneficial in trauma situations, especially during the pre hospital 'golden hour' period

which has a large impact on outcome for the patient, and during which blood is usually not available, or during which transfused blood has not yet reached its full effectiveness. Fluorocarbon-in-water oxygen-delivering emulsions also have potential for the treatment of conditions of restricted blood flow such as those which occur in myocardial infarction or stroke; for the prevention of ischemia during the percutaneous transluminal coronary angioplasty procedure; in cardioplegic solutions to protect the heart during cardiopulmonary bypass surgery; during extracorporeal circulation; as reperfusion solutions to prevent reperfusion injury; to improve oxygen delivery to certain tumors, making the tumor more responsive to radiation and chemotherapy; and for organ and tissue preservation, etc. These so-called 'blood substitutes'are the most publicized of the fluorocarbon-based products destined for therapeutic use; In addition to the fluorocarbon-in-water emulsions, a neat fluorocarbon is currently in clinical trials for the treatment of the respiratory distress syndrome by liquid ventilation (Riess JG et al.,1998). Fluorocarbons are also used as ocular tamponades for managing complicated retinal detachments (Chang S et al.,1989). Diverse gaseous microbubbles, generated or stabilized by flurocarbons, are in advanced stages of development as contrast agents for ultrasound imaging (Mattrey et al.,1994). Fluorinated vesicles (liposomes) and other self-assemblies of fluorinated amphiphiles show promise as containment systems for the delivery and targeting of an extensive range of drugs, prodrugs, contrast agents, vaccines, genetic material, etc (Riess 1994).

1.15.7 ***PFCs as Cell culture media supplements****:* There is a growing interest in the use of PFCs for regulating respiratory gas supply to cultured cells, and this aspect was recently

reviewed in detail (Lowe et al.,1998). Such use of PFCs in culture systems can reduce or eliminate cellular damage caused by conventional, and more vigorous, aeration methods (i.e.) stirring, leading to improved cell growth. This approach has been applied to studies with human cells, including neonatal foreskin fibroblasts and retinoblastoma cells,which were grown successfully at an interface formed between PFC liquid and aqueous culture media. PFCs have enoromous potential in cell biotechnology where, through their facilitation of gas supply, they stimulate cell division and biomass production, leading, in some instances, to increased production of valuable cellular products. PFCs are especially advantageous for use in cell and tissue culture systems, since they are heat stable, readily sterilized (e.g. by autoclaving) and can be recovered from aqueous systems with the potential for recycling, thus making them economically viable.

1.15.8 ***PFCs in Hybridoma culture****:* As a pressing need exists for better oxygen supply techniques in cell culture. Perfluorocarbon emulsions were applied to hybridoma cultures grown in tissue culture tubes and column bioreactors. The oxygen transfer enhanacement effect of perfluorocarbon emulsions was clearly demonstrated by the higher cell densities obtained in emulsion-supplemented systems. In addition, perfluorocarbon emulsions were shown to provide better cell suspension in a low-shear environment. Researchers took advantage of PFCs high oxygen carrying capability and passed the oxygenated emulsions through the cultivation systems for supplying oxygen to cells (Jensen, M.D 1987). Although some used PFC emulsions as liquid microcarriers (Giaever, I et al.,1983, Keese,C.R et al.,1983), other researchers took advantage of their high oxygen carrying capability and passed the oxygenated PFCs of PFC emulsions through the cultivation

systems for supplying oxygen to cells (Aldercreutz et al.,1983, Damiano,D et al.,1985, Hamamoto,K.,1987).While higher oxygen transfer rates were shown to be achievable, several disadvantages of applying pure PFCs in cell culture can be expected. Substitution of fluorine for hydrogen leads to increase in molecular mass and high densities (ranging from 1.7-2.0 g/cc), pure PFCs will settle rapidly to the bottom of a vessel when applied to culture systems with no or only mild mixing. The efficiency of using pure PFCs as oxygen transfer enhancers in low-shear cell culture will therefore be unacceptably low due to the small interfacial areas available to oxygen transfer between gas and PFC phases and between PFC and aqueous phases. Furthermore, it was clearly identified that there is a problem of protein stripping from the growth medium by the use of pure PFCs (Ferro, J.R et al.,1986). In the work of Lu et al.,1992, the advantages of applying PFC emulsions in cell culture were first analyzed conceptually and then investigated experimentally with hybridoma cultures grown in surface aerated tissue culture tubes and column bioreactors. With very fine PFC droplets (<0.2μm), the emulsions remain suspended in a very low-shear environment and provide a high PFC/water interfacial area for oxygen transfer. PFC molecules enables gaseous oxygen to enter the spaces between them. The protein-stripping problem was also avoided since the active surface of PFC droplets was already covered by the surfactant molecules.

Advantages of Applying PFC Emulsions in Cell Culture: The application of PFC emulsion can benefit cell growth and produce a higher yield in cell culture in two ways.1) they can enhance the oxygen supply to the cells and 2) prevent cells from settling out of suspension due to inadequate mixing, that is often found in cell culture vessels (Lu- et al., 1992).

Enhanced oxygen supply to cells: The properties of perfluorochemical liquids, particularly their high gas solubility, enables them to be exploited in cell biotechnology. They can facilitate respiratory-gas delivery to prokaryotic and eukaryotic cells in the culture. In some systems, they can stimulate production of biomass, yields of commercially important cellular products. Lu et al.,1992 used emulsified FC-40 to enhance the oxygen supply to monoclonal-antibody- producing hybridoma cells cultured in surface-aerated rotating tubes. In this study, an increase in the density of viable cells occurred when the culture medium was supplemented with 10% (v/v) of FC-40 emulsion, consistent with theoretical predictions of enhanced oxygen transfer. The authors reported that the exponential-growth phase was prolonged, with a greater-than-sixfold increase in the population of viable cells in PFC-emulsion-supplemented systems. They suggested that the higher oxygen- transfer rates satisfied the increased oxygen demands of the greater cell populations. In bioreactor cultures of the same hybridoma cells, there was also evidence that the emulsion protected cells against mechanical damage. FC 40 is chemically perfluorotributylamine $(C_4F_9)_3N$.

Synergistic effects of PFCs and surfactant supplements: The surfactant lowers the interfacial tension and, possibly, acts as a cell-protecting or growth-enhancing agent (Lowe, K.C et al., 1993). The addition of Pluronic® F-68 to the aqueous culture medium was sufficient to reduce the PFC-water-interfacial tension by approximately 40%, thus facilitating maximum contact of protoplasts with the interface.

PFCs have multifaceted applications in both prokaryotic and eukaryotic cell-culture systems. Their key physical property is to increase the respiratory-gas supply, thus

increasing biomass production and, in some systems, the yields of commercially important cellular products.

1.16 Growth Monitoring

The progress of suspension cultures is monitored via pH, oxygen, CO2 and glucose electrodes that read from the culture *in situ*, and by assaying the utilization of nutrients, such as glucose and amino acids, or the buildup of metabolites, such as lactate and ammonia, and products, such as immunoglobulins from hybridomas. The number of cells and other parameters, such as protein, are determined in samples drawn from the culture and are used to calculate the total biomass. Even NMR analysis can be performed to facilitate the growth monitoring (Freshney I 2000).

1.17 Non-Producing Cell Lines-Possibilities of low yields

Genetic instability: The characteristics of a cell line do not always remain stable. Particularly the mouse cell lines are genetically unstable and transform quite readily. Continuous cell lines, particularly from tumors of all species, are very unstable, not surprisingly, as this instability was a major reason for their undergoing the necessary mutations to become continuous (Freshney I 2000). There are two main causes of genetic variation: (1) The spontaneous mutation rate appears to be higher *in-vitro*, associated, perhaps, with the high rate of cell proliferation, and (2) mutant cells are not eliminated unless their growth capacity is impaired.

Chromosomal Aberrations: The method of establishing permanent cell lines capable of producing antibodies directed to predefined immunogen is based on the fusion of immune

lymphocytes with myeloma cells adapted for growth in tissue culture conditions. Several genetic processes occur during and after cell fusion. The genes may continue to be rearranged after the fusion and chromosomal losses have been reported over longer periods of time (Hunkeler et al.,1989).

1.18 *In-vitro* humane alternatives to the ascities production of Mabs

Traditional route of Mab production is usually by the ascities *in-vivo* method, where the hybridoma cells are injected into the peritoneal cavity of the mice causing ascitic tumors (Goding,1980). This results in the hybridoma cell propagation and the Mab is secreted into the peritoneal cavity. The ascities fluid is then harvested and purified by affinity chromatography to obtain the pure Mab.

In the past decade, after the discovery of hybridoma technology, millions of animals have suffered and were sacrificed, inspite of the humane alternatives that are available. The government legislation and the ethical issues on the laboratory animal care have forced the implementation of rules and Massachusetts society for medical research has formulated the Three R's" Reduction, Refinement and Replacement principles in the interests of the animal safety. Reduction refers to the use of fewer animals than previously used. Refinement means alteration of existing procedure to minimize the discomfort they cause to the animals. Replacement means the use of the research methods or models that do not use animals instead of methods that do. Several European countries have implemented the restrictions on the ascities mode of antibody production. As an outcome of the Netherlands government Code of Practice for the Production of Monoclonal antibodies (Anon 1989), the Dutch scientists have encouraged the *in-vitro*

methods of Mab production. Similar restrictions have been imposed in Germany, Sweden, Switzerland and United Kingdom. In United States, IACUCs (Institutional Animal Care and Use Committees), USDA (U.S department of agriculture), HSUS (Humane Society of the United States), AWA (Animal welfare act) are the key players.

The European Union scientific representatives met in October 1996 at the European Center for the Validation of alternative methods to review the current status of *in-vitro* and *in-vivo* routes of monoclonal antibody production. The conclusion of the meeting was that " for all levels of Mab production, there are one or more *in-vitro* methods which are not only scientifically acceptable, but are also reasonably and practically available; and as a consequence, *in-vivo* production can no longer be justified and should cease " (Marx, et al., 1997). The group has called upon the Europe wide prohibition on the ascities method of Mab production. The response is the adoption of *in-vitro* methods at all stages of the Mab production.

The three principal steps in the production of monoclonal antibodies are :

1) Immunization 2) Hybridoma generation and 3) Mab production. Each of these steps have their own potential for alternative method adaptation. This is presently carried over as an *in-vivo* procedure using mice, however this can be replaced with the cell culture. However, the immunization has always been as a *in-vivo* method, but the hybridization is always an *in-vitro* method. The final production of the monoclonal antibody can be from either the ascities or the *in-vitro* alternatives.

Mabs obtained from the *in-vitro* methods exhibit immunoreactivity ranging from 90-95 %, and this is significantly higher than the Mabs produced by ascities (John Mc Ardle,1998).

1) Much higher percentage of the mice do not produce antibodies,where as 3% or less of the *in-vitro* methods fail (John Mc Ardle,1998).
2) The quality of the *in-vitro* Mabs is equal to or better than that derived from the in vivo methods (John Mc Ardle,1998).
3) Glycosylation patterns can be easily regulated *in-vitro*, since with ascities, they can vary between each individual animal. Glycosylation is a factor because it has the influence on the antigen-binding capacity and the resistance of an antibody to proteolysis (John Mc Ardle,1998).

The further drawbacks of ascities which is subject to criticism are the disadvantages such as: 1) Suffering and cruelty to animals; 2) Ascitic fluids may be contaminated with the murine plasma proteins, immunoglobulins (decreases the immunoreactivity), infectious agents and bioreactive cytokines (John Mc Ardle,1998); 3) the need for proper animal facilities and daily monitoring of the animal facilities (John Mc Ardle,1998) 4) mice only produce Mabs for a few days (John Mc Ardle,1998); 5) some hybridomas are difficult to grow in mice (John Mc Ardle,1998); 6) 60-80 % of the mice may not produce ascities due to premature death, development of solid tumors, or failure to establish the *in-vivo* hybridoma growth (Kuhlman, I.,1989).

It is revealed from the human clinical experience that the growth of abdominal tumors is very painful. Further, ascities fluid accumulation is associated with abdominal distension, anorexia, nausea, vomiting, respiratory distress, edema, decreased mobility and fatigue (John Mc Ardle, 1998). Mice used for the ascites accumulation of Mabs frequently exhibit a range of symptoms including: (1) Roughened haircoat, hunched posture, abdominal distension, anorexia, cachexia, anemia; (2) decreased

activity and body mass, dehydration, shrunken eyes; (3) difficulty walking, respiratory distress due to an elevated diaphragm; (4) circulatory shock due to excessive fluid removal; (5) decreased venous, arterial and renal blood flow; (6) classical peritonitis; (7) immunosuppression associated with adjuvant use; and (8)up to 20% mortality after removal of ascitic fluid. It is not uncommon for fluid to be withdrawn in amounts greater than the entire blood volume of the animal. These symptoms become increasingly severe the longer the animals are allowed to survive (Jackson, L.R.et al.,1996). Pathological changes associated with ascites production of Mabs are known for each step in the process. Use of adjuvants produces mild to severe peritonitis and inflammation. Fluid removal may cause hemorrhage, edema and death. As expected, growth of the ascitic tumors creates a variety of responses including: (1) adhesion of the abdominal wall, bladder, diaphragm, kidneys, liver, seminal vesicles, testicles, and ureters; (2) linear lesions in diaphragm muscles; (3) enlarged thoracic lymph nodes and lymphatic obstruction; (4) tumors with extensive hemorrhagic and necrotic areas; (5) disseminated tumors in mesenteric, lumbar, kidney, and testicular regions; (6) centro-lobular liver necrosis; and (7) solid tumors throughout the abdomen (Jackson, L.R.,et al.,1996).

From the above, it is obvious that animals used for ascites production of monoclonal antibodies are routinely subjected to chronic pain and distress. Use of adjuvants further complicates this situation by injuring the animals before the process begins (John Mc Ardle1998).

1.18.1 In-Vitro Mab Production

The most relevant type of *in-vitro* alternative for each research of diagnostic situation depends largely on the quantity and purity of monoclonal antibody needed.

There are, however, some criteria for rating each system. The ideal method (Falkenberg, F.W. et al.,1993) would have the following properties:

1. Should have expendable material cost similar to that of a mouse.
2. Should be a simple technique, requiring no special expertise beyond that for typical cell culture procedures.
3. Should not require prior adaptation of the hybridomas or special culture conditions.
4. Should have significantly higher concentration and quality of Mabs than for simple stationary cultures.
5. Should be a closed, reusable system; free of contamination.
6. Should be affordable to all laboratories.
7. Should produce adequate quantities of Mabs in a reasonable period of time.

Only few *in-vitro* methods have so far met these criteria.

1.18.2 Commercial Scale Production of Mabs

The cultivation of hybridoma cells for the production of a wide variety of therapeutic agents has expanded rapidly over the past several years. As the need for increasing amounts of these products has grown, new and larger-scale bioreactors have been examined for cultivation of hybridomas. Once suitable hybridomas have been produced, it is essential to scale up the production of antibodies to achieve an appropriate yield. Two basic and quite different strategies can be used. One is to use large-scale vessels (bioreactors) in a manner analogous to the procedures used in fermentation technology to produce such desirable compounds as antibiotics from microbial cell

culture. The culture conditions are easily controlled and maintained, yields are fairly predictable, downstream processing is fairly straightforward and a standardised product is made. With improvements in animal cell technology the general move is towards producing monoclonal antibodies in bioreactors rather than from ascites tumors.

1.18.3 Classification and types of Bioreactors

There are a number of factors that are typically used to distinguish among bioreactor types.

1. Batch bioreactor

A batch reactor has no inlet or outlet flows. In operation, the bioreactor is charged with reactants, the reaction proceeds, and then the reactor is emptied. They are often used where small quantities of very expensive products are produced.

2. Homeostatic Culture Environment

To overcome the extreme culture conditions of "feast or famine"inherent in closed batch culture, various approaches have been used, all directed at establishing a steady-state culture environment. These include a) fed batch and b) continuous chemostat and continuous cytostat.

2(a) Continuous Culture

In this mode, the fresh medium is added continuously to the growth vessel and spent medium and cells are removed from the vessel at the same rate. To maintain the culture in continuous culture mode, you need to maintain the system either in the chemostat or the biostat mode. The most practical continuos culture type is the chemostat. The concept is that the nutrient medium contains saturating concentrations of all components required for cell growth, with the exception of one, the concentration of this component and the feed rate will determine the cell density and growth rate of the culture. In the chemostat,

the growth rate is determined by the rate of medium addition and the growth yield by the concentration of the growth-limiting nutrient (usually the carbon source). In biostat type of culture system, the cells are grown to the mid-log phase (monitored by daily cell counts), a measured volume of cells is removed each day, and replaced with an equal volume of medium. Alternatively, the cells may be run off continuously, at a constant rate, at mid-log phase, and medium added at the same rate. The objective is to keep the culture conditions constant rather than to produce large number of cells.

Various mammalian cells, however, have been grown in continuous chemostat culture, often under glucose limitations. This does provide an efficient continuous system for production of cells and derived products in a homeostatic culture environment.

2(b) Fed-Batch-type

Fed-Batch culture is characterized by a continuous feed of medium or a specific limiting nutrient to a culture of cells in a closed system at a rate that maintains a constant nutrient level. Productivities of much fermentation are enhanced by addition of various nutrients, such as a carbon or nitrogen source, during the fermentation, resulting in a fed-batch type process. Similarly, since metabolic products accumulate, some of which (e.g., ammonia) might be toxic, the medium feed rate must be controlled to dilute these effects.

3. Semi-Batch bioreactor

The term semi-batch reactor is often applied to reactors that operate in neither fully continuous nor batch mode. The reactor may initially contain a mass of material, and, during the course of reaction, material is added, but none is removed. After some time, the addition might be stopped and the products removed. Alternatively, once the addition of material stops, the reactor may continue to operate as a batch reactor. The semi-batch reactor is a transient reactor, and cannot operate at steady state.

1.18.4 Different types of Laboratory Scale Bioreactors in use

Several types of bioreactors have been developed for Mab production. They are:

a) Culture bags-eg I-Mab, TC-Tech

b) Rotating chambers-eg Miniperm, Techne permeable tube bioreactor

c) Perfused Suspension Culture-eg Hollow fiber bioreactors

d) Cell suspension bioreactor-eg NASA Bioreactor

e) Multisurface Propagators-eg Nunc Cell Factory

f) Roller culture bottles

g) Microcarriers

h) Fluidized-Bed and Fixed bioreactors

i) Microencapsulation

j) Membrane based tissue culture flasks-eg CL-1000

The bioreactors such as the Culture bags, Roller culture, Membrane based tissue culture flask, Rotating chamber bioreactors are discussed in detail in the later chapters.

- **Microcarriers**

Suspension cells can be trapped in the interstices of the bead matrix. Monolayer cells may be grown on microbeads ~150μm in diameter and made of plastic, glass, gelatin, or collagen (Griffiths, 1992). Culturing monolayer cells on microbeads gives a maximum ratio of the surface area of the culture to volume of the medium, up to 90,000 cm^2/L, depending on the size and density of the beads, and has the additional advantage that the cells may be treated as a suspension.

- **Cell Suspension –Non sedimenting Bioreactor**

This Bioreactor was developed by NASA. Intrigued by the concept of growing cells in zero gravity, NASA constructed a rotating chamber in which cells, growing in suspension, achieved simulated zero gravity by slowly rotating the chamber, altering the sedimentation vector continuously.

- **Multisurface Propagators**

The simplest system for scaling up monolayer cultures (anchorage-dependent) is the Nunclon Cell Factory. This system is made up of rectangular Petri-dish-like units, with a total surface area of 600-24,000 cm^2, interconnected at two adjacent corners by vertical tubes. Because of the positions of the apertures in the vertical tubes, medium can flow between compartments only when the unit is placed on end. The cell factory has the advantage that it is not different in the geometry or the nature of its substrate from a conventional flask.

- **Fluidized Bed reactors**

In fluidized bed reactors, porous beads of a relatively low density –made of ceramics, or a mixture of ceramics, or a mixture of ceramics and natural products, such as collagen-are suspended in an upward stream of medium when the flow rate of the medium matches the sedimentation rate of the beads. While suspension cells lodge in the beads by entrapment, monolayer cells attach to the outer surfaces as well as the interstices of the porous bead.

- **Fixed-Bed Reactors**

Systems have also been developed with beds of glass beads, with the medium being perfused upward through the bed or percolating downward by gravity. The product is collected with the spent medium in a reservoir. Cells grown on the surface of beads are perfused with medium.

- **Microencapsulation**

Sodium alginate behaves as a sol or gel, depending on the concentration of divalent cations. It will gel as a hollow sphere around cells in suspension in a high concentration of divalent cations. Because the alginate acts as a barrier to high-molecular-weight molecules, macromolecules secreted by the cells are trapped within the vesicle, while nutrients, metabolites, and gas freely permeate the gel. The product and cells are recovered by reducing the concentration of divalent cations. These gels have a low immunoreactivity and can be implanted *in-vivo*.

1.19 Product Formation

Antibody production does not appear to be feedback inhibited, except for cell lines that produce very low levels (<1 mg/L) of antibody (Merten et al.,1985). This is consistent with results showing essentially constant specific antibody production rates for a 10-fold increase in antibody concentration at the same growth rate in continuous suspension culture (Fazekas 1983). Hybridomas are often unstable with respect to antibody production and must be periodically recloned to select for highly producing cells. Loss of antibody productivity has been observed during extended periods in continuous culture (Miller 1987; Hu et al.,1987). This could be due to lower production by all cells or to a decrease in the fraction of cells producing antibody. Significant antibody production often occurs after the viable cell concentration has started to decline in batch culture. Miller et al.,1987 observed that the specific (per viable cell) antibody production rate in continuous culture was higher at lower cell viabilities. Increased specific antibody production rates at low cell viabilities suggest that hybridoma cells may increase antibody synthesis in response to stress. All cells are known to increase the

production of heat shock proteins in response to elevated temperatures and other stress factors (Schlesinger et al.,1982).

1.20 Down stream Processing of Mabs

There are various methods by which the Mabs are purified using the various purification techniques available. Among all the techniques, affinity chromatography stands as the most versatile technique feasible for the down stream processing of the Mabs.

Ammonium Sulfate Precipitation: High salt concentration (i.e. small and highly ionic molecules) removes water molecules interacting with Ab, decreasing solubility of proteins, yielding a precipitate.Various concentrations of ammonium salt precipitates out different proteins dependent on their size and charge (as well as the species of Ab source, pH, temperature, number and position of polar groups on the protein). A 33-50% ammonium sulphate saturation is a standard concentration used to precipitate immunoglobulins (Hardy,R.R.1986) .

Caprylic Acid (Octanic Acid): Weak acidic buffer plus the addition of short chain fatty acids (caprylic acid) will precipitate most serum proteins except IgG in the supernatant. This is an inexpensive technique where it needs only 0.75-mL caprylic acid for 10-mL mouse serum (Sterogene Bioseparations Inc). The concentration of the caprylic acid used for the purification stands patented by Sterogene Bioseparations Inc

DEAE (Diethylaminoethyl) Cellulose/Sepharose: Separates proteins on the basis of surface charge. Proteins electrostatically bind onto a matrix bearing the opposite charge. Proteins are eluted by a change in the pH or increase in the concentration of the buffer. Anion exchange resins are used for positive and negative selection of Ab. Ab has a basic

isoelectric point when compared to most of the proteins in serum. When the pH of the Ab solution is lowered, the Ab elutes off the column (negative selection in batch quantities). Increase in the pH of the Ab solution causes the Ab to bind to the column (positive selection) (Ellis H 1978). Normally used after ammonium sulfate precipitation (for IgG).

Gel Filtration Chromatography: Separates proteins on the basis of molecular size using porous beads of cross-linked dextran or agarose. A molecular sieve of defined pore size sifts for the molecular weight range of interest. A neutral wash buffer is used to elute Ab. Decent yield for IgM, yet poor yield for IgG.

Thiophilic Gel: Thiophilic absorbent is a modified sulfur containing silica bead that has a high affinity to all IgG subclasses in a high salt environment. Especially used for purification of the bispecific Mabs (Kreutz et al.,1998).

Immunoaffinity Purification: Covalently bound antigen to a support matrix (eg. activated sepharose, magnetic beads) is used to purify Ab (Axen,R et al.,1967). Ab is eluted by changing the pH or increasing the ionic strength of the elution buffer. Ab purified is specific to the antigen. Antigen must be able to withstand harsh elution conditions has the ability to renature after equilibration so that you can reuse Immunoaffinity matrix (Ellis H 1978). Monoclonal Ab with low affinity to antigen may wash off with the rest of the unbound proteins.

1.20.1 Affinity Chromatography: Affinity chromatography occupies a unique place in separation technology since it is the only technique which enables purification of almost any biomolecule on the basis of its biological function or individual chemical structure. Although the technique became routinely available little more than ten years ago, its

application has developed so rapidly that it is now used in almost every laboratory concerned with the purification of biological substances. The widespread adoption of affinity chromatography in such a short time reflects its success in achieving rapid separations which are time-consuming, difficult or even impossible using more conventional techniques.

Affinity chromatography is a type of adsorption chromatography in which the molecule to be purified is specifically and reversibly adsorbed by a complementary binding substance (ligand) immobilized on an insoluble support (matrix) (Axen,R1967). Purification is often of the order of several thousand-fold and recoveries of active material are generally very high. Many spectacular separations have been achieved in a single step allowing immense timesaving over less selective multi-stage procedures (Pharmacia fine chemicals, Uppsala, Sweden). Affinity chromatography has a concentrating effect, which enables large volumes to be conveniently processed. The high selectivities of the separations derive from the natural specificities of the interacting molecules. For this reason, affinity chromatography can be used for

- Purifying substances from complex biological mixtures
- Separating native from denatured forms of the same substance
- Removing small amounts of biological material from large amounts of contaminating substances.

The principle of affinity chromatography is shown diagramatically in the figure1.3. A successful separation requires that a biospecific ligand (L) is available and that it can be covalently attached to a chromatographic bed material, the matrix. It is also important that the immobilized ligand retains its specific binding affinity for the substance of

interest (S) and that methods are available for selectively desorbing the bound substances in an active form, after washing away unbound material. Any component can be used as a ligand for purifying its respective binding substance. Biological systems in which affinity chromatography is most frequently used are listed in table 1.2

Table: 1.2 Examples of substrate and ligand for the affinity chromatography

Ligand	**Substrate**
Enzyme :	Substrate analogue, inhibitor, co-factor
Antibody:	Antigen, virus, cell
Lectin:	Polysaccharide, glycoprotein, cell surface receptor, cell
Nucleic acid :	Complementary base sequence, histone, nucleic acid polymerase,
	binding protein
Hormone, vitamin:	Receptor, carrier protein
Cell:	Cell surface specific protein, lectin

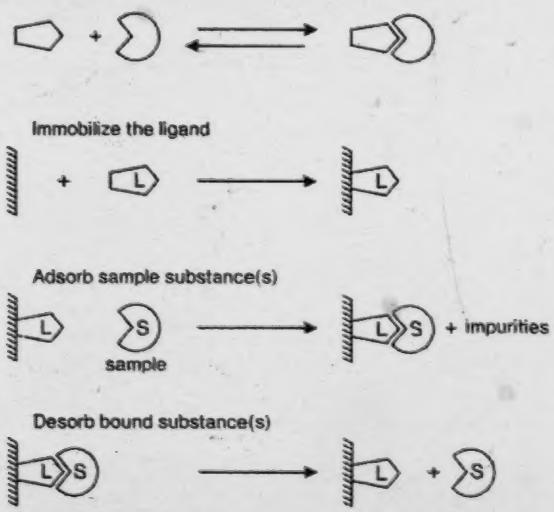


Figure 1.3 Principle of affinity chromatography

1.20.1.1 Building blocks of Affinity Chromatography

The matrix: Matrix is the support to which the ligands are immobilized. Sepharose is the most widely used matrix. Sepharose is the bead formed agarose gel, which displays virtually all the features required of a successful matrix for immobilizing biologically active molecules. The hydroxyl groups on the sugar residues can be easily derivitized for

covalent attachment of a ligand. The open-pore structure makes the interior of the matrix available for ligand attachment and ensures good binding capacities, even for large molecules. The bed-form of the gel provides excellent flow properties with minimal channeling in the bed, which ensures that rapid separations are obtained.

The Ligand: The selection of the ligand for affinity chromatography is influenced by two factors. Firstly, the ligand should exhibit specific and reversible binding affinity for the substance to be purified. Secondly, it should have chemically modifiable groups which allow it to be attached to the matrix without destroying its binding activity.

The ligand should ideally have an affinity for the binding substance in the range 10^4 to 10^8 M in free solution. Interactions involving dissociation constants lesser than 10^4M, for example the binding reaction between an enzyme and a weak inhibitor, are likely to be too weak for successful affinity chromatography. Conversely, if the dissociation constant is greater than approximately 10^8 M, for example the affinity between a hormone and hormone receptor, elution of the bound substance without inactivation is likely to be difficult.

Spacer arms: The active site of a biological substance is often located deep within the molecule and adsorbents prepared by coupling small ligands (e.g. enzyme cofactors) directly to sepharose can exhibit low capacities due to steric interference between the matrix and substances binding to the ligand. In these circumstances a "spacer arm" is interposed between the matrix and ligand to facilitate effective binding. If the ligand is relatively small, it is generally preferable to incorporate a spacer arm between ligand and matrix, allowing the immobilized ligand to interact with the protein. The length of the spacer arm is critical. If it is too short, the arm is ineffective and the ligand fails to bind

substances in the sample. If it is too long, non-specific effects become pronounced and reduce the selectivity of the seperation. O'Carra et al.,1973, have demonstrated that very long spacer arms can bind substances in the sample by hydrophobic interactions. Non-specific hydrophobic interactions are undesirable in affinity chromatography. However, the interaction between immobilized hydrophobic groups and hydrophobic regions allows proteins to be separated on the basis of their differences in hydrophobicity.

Coupling Gels: Methods are available for immobilizing ligands quickly, easily and safely through a chosen functional group. The correct choice of coupling method depends on the substance to be immobilized (Axen,R et al.,1967). The following derivatives of Sepharose allow the convenient immobilization of ligands without the need for complex chemical syntheses or special equipment:

1. CNBr-activated Sepharose – enables ligands containing primary amino groups to be safely, easily and rapidly immobilized by a spontaneous reaction.

2. Epoxy-activated Sepharose has a long hydrophillic spacer arm and provides a method for coupling through hydroxyl, amino or thiol groups.

3. Activated Thiol Sepharose has a glutathione spacer arm and provides a method for reversibly coupling proteins through free thiol groups.

4. Thiopropyl-Sepharose has a short hydrophillic spacer arm and provides a method for reversibly coupling proteins and small thiolated ligands through thiol groups; it also reacts with heavy metal ions, alkyl and aryl halides undergoes addition reactions with compounds containing C=O, C=C and N=N bonds.

Group Specific adsorbents: Group specific adsorbents have affinity for a group of related substances rather than for a single substance. The same ligand can therefore be used to

purify several substances without the requirement that a new adsorbent be prepared for each different substance to be purified (Ellis H 1978). Within each group of adsorbed substances there is either structural or functional similarity. The specificity in group specific (general ligand) affinity chromatography derives both from the selectivity of the ligand and the use of selective elution conditions. For example Protein A-Sepharose, ProteinG-Sepharose, have specificity or affinity towards the Fc region of IgG and related molecules, Biotin-agarose has affinity towards the anti-biotin antibodies. Protein L-Sepharose has specificity towards the light chain of the antibody.

1.20.1.2 Elution: Elution methods may be either selective or non-selective in nature. Usually, non-selective elution methods are applied in combination with group specific adsorbents and non-selective elution methods are used in combination with highly specific adsorbents. A compromise may have to be made between the harshness of the eluent required for elution and the risk of denaturing the eluted material. Forces that maintain the complex include electrostatic interactions, hydrophobic effects and hydrogen bonding. Agents which weaken these interactions may be expected to function as efficient eluting agents. Elution may be achieved by either a step-wise or a continuous change in conditions. Continuous gradients are useful for their zone sharpening effect; the trailing edge of a desorbed substance experiences a higher concentration of eluting agent than the peak and is therefore made to move faster down the column than the main peak. When substances are very tightly bound to the adsorbent, it may be effective to stop the flow after applying eluent and to allow the adsorbent to remain in the presence of eluting agent (30min to 2hr is commonly used) before commencing elution. This allows

dissociation to take place prior to elution and thus ensures good recoveries of adsorbed substances.

pH change: A pH shift is sufficient to elute the adsorbed substances. The change in pH changes the degree of ionization of charged groups at the binding sites. This is usually reflected in the change of behavior of the system in free solution i.e. a change in the ability to form a complex. Binding takes place mainly around physiological pH so desorption is generally effected by a decrease in pH. The chemical stability of the matrix, ligand and adsorbed substances determines the limit of pH, which may be used.

Ionic strength: When using a change in ionic strength to effect desorption the similarities to ion exchange chromatography is apparent. For elutions with ionic strength, a buffer with increased ionic strength is used. Gradient or step-wise elution may be used. A gradient of increasing salt concentration can be used to separate substances bound to the adsorbent. NaCl is most often used. If the interaction has a very high affinity ($Kd>10^6 M$), a chaotropic salt may be required. Chaotropic ions disrupt the structure of water and reduce hydrophobic interactions and are therefore effective desorbing agents.

Affinity elution: Selective eluents are often used to separate substances on a group specific adsorbent and also when binding affinities are relatively low (Kd 10^4 to 10^6 M). Selectively retained substances are usually displaced at low concentrations of eluting agent, often less than 10mM. The eluting agent either competes for binding to the adsorbed substance or for binding to the ligand. Substances may be separated by either a concentration gradient of a single eluent or by pulse elution using several desorbing agents (Lane,D ed 1998).

1.20.1.3 Advantages of Affinity Chromatography: Affinity chromatography offers the user several major advantages compared to other protein purification techniques. It is important these advantages are emphasized. The advantages are truly remarkable compared to conventional purification methods such as ion exchange, hydrophobic interaction and gel filtration. The advantages may be summarized as:

Selective Binding and Elution

- Gives very pure product in a single step
- Extremely high levels of purification can be achieved, several thousand-fold in a single step
- Provides high yields of purified product
- Massively reduced processing time and cost
- High concentrations of material leaves the column
- Large scale use

Targeting the binding and elution of a specific protein results in very high degrees of purification in a single step. Conventional purification methods generally give much lower levels of yield and purification as a function of using a series of separation steps. Material is, of course, lost at each step, resulting in appreciable losses on completion of the purification regime. Conversely, affinity chromatography yields very pure protein in a single step with minimum wastage. Consequently, affinity chromatography is the most economic process possible. Apart from high yield/purity the total processing speed is a major factor in reducing labor and capital costs (Lane,D ed 1998).

1.20.1.4 Dye-Ligand Affinity Chromatography: Dye-ligand affinity chromatography is performed with an adsorbent consisting of a solid support matrix to which a dye has been

covalently bonded. A class of textile dyes, known as reactive dyes, are commonly used for protein purification purposes since they bind a wide variety of proteins in a selective and reversible manner. They are easily immobilized to polysaccharide-based support matrices, such as beaded agarose, dextran and cellulose. From a chromatographic viewpoint, synthetic affinity ligands, such as reactive dyes, are preferable to biological ligands (e.g. antibodies) since the former are relatively inexpensive and are more resistant to chemical and biological degradation. Dye-ligand affinity chromatography generally gives higher degrees of purification compared to low-specificity techniques, such as ion exchange, hydrophobic interactions and gel permeation chromatography (Scopes,R.K 1986, Kroviarski,Y., et al 1988).

1.21 Enzyme Linked Immunosorbent Assay- ELISA

The use of antibodies for the identification and measurement of analytes is called immunoassay. The name ELISA has derived from the use of an enzyme conjugate as a key reagent in the signal generation of the assay.

History and Development of ELISA: Enzyme-immunoassays (EIA) evolved as a result of the findings by Nakane and Pierce (1966) that antibodies could be labeled with enzymes for use in histochemical staining procedures, and by Catt and Tregnar (1967) who described solid-phase radioimmunoassays (RIA). The substitution of enzyme labels for radioactive ones in the solid-phase RIA resulted in solid-phase EIA tests for human chorionic gonadotropin (Van Weeman and Schuurs, 1971) and for IgG detection (Engvall and Perlmann, 1971). In 1971 Engvall & Perlmann and Van Weeman & Schuurs working independently were the first to report the development of ELISAs. Engvall & Perlmann

have developed their assay with alkaline phosphatase and Van Weeman & Schuurs with horseradish peroxidase. The latter authors coined the term "enzyme-linked immunosorbent assay (ELISA)" for solid phase EIA tests. Initial solid-phase EIA tests were not as sensitive as the corresponding RIA, but improvement in enzyme-labeling techniques have made the two types of assays comparable for detecting a number of antigens and antibodies. In some systems where RIA and EIA have been directly compared, there is little difference in the sensitivity or specificity of the two assays (Sarkkinen et al.,1981). The advantages of enzyme labels over radioactive ones are mainly convenience in use, in that the labeled immunoreagents are stable for long periods, and the precautions and disposal procedures required for radioisotopes are not required. In addition, the use of chromogenic substrates for the enzyme labels permits visual interpretation of test results in some cases. The only real disadvantages of EIA tests are the loss of antibody reactivity that may result from conjugation to enzymes, and the limits of substrate detection. For example, use of enzymes that have molecular weights higher than that of IgG molecules such as β-D-galactosidase (MW 540,000) can cause stearic hindrance of antibody activity (Herrmann and Morse 1974).

The use of monoclonal antibodies over polyclonals in EIA tests offers two potential advantages: (1) Improved specificity (2) Improved sensitivity.

Principle: The basic principle of an ELISA is to use an enzyme to detect the binding of Ag and Ab. The enzyme converts a colorless substrate (chromogen) to a colored product, indicating the presence of Ag:Ab binding. An ELISA can be used to detect either the presence of Abs or Ags in a sample, depending on how the test is designed.

1.21.1 Overview of Immunoassay Technology: ELISA has replaced a number of more cumbersome and time consuming "classical" serological techniques, and has also widened the scope of the detection methods for viruses, bacteria etc and their related markers of infection. Immunoassays utilize the specific reaction between an antigen and an antibody and is applicable to any molecule that can elicit an antigenic response directly (e.g.protein) or indirectly (e.g. haptens coupled to a protein carrier). Immunoassays are now widely used for the specific and quantitative measurement of a variety of analytes. To enhance the sensitivity of immunoassays, labels such as enzymes, radioactivity, fluorescent and luminescent compounds are linked directly or indirectly to either the analyte or antibody. The signals from the labels are used to reveal and amplify an antibody-antigen reaction. There are two basic types of ELISA techniques based on the steps involved. a) Homogeneous and b) Heterogeneous

a) Homogenous assays are completed in one step, with all reagents added simultaneously without separation of the bound and free reactants.
b) Heterogeneous assays are completed in more than one step, in which various reagents are added sequentially and generally includes at least one wash step to reduce the bound and free reactants.

The many immunoassay formats described can be classified into two types based on whether the reaction design uses competitive (EIA) or non-competitive methods (ELISA).

i) Competitive assay or Direct assay (EIA): This assay uses a single antibody to measure small molecules. The assay works on the principle that two reactants labeled and unlabeled analyte, "compete"for binding to the limited number of antibody sites. An

affinity-purified capture antibody (typically anti-IgG) is pre-coated onto a microplate. A limited concentration of primary antibody and enzyme-linked analyte along with the sample are added simultaneously. Sample analyte and enzyme-linked analyte compete for the limited number of binding sites on the primary antibody. The substrate when added is hydrolyzed by the enzyme, thereby producing a color product that can be measured. The amount of labeled analyte bound is inversely proportional to the amount of unlabeled analyte present in the sample (signal decreases as analyte concentration increases). Substrate hydrolysis product is inversely proportional to the amount of analyte present.

ii) Non–Competitive assay or indirect assay (Sandwich ELISA): ELISAs work on the principle that two antibodies "sandwich" the analyte to be measured. This format is typically used to measure large molecules. Both the capture and detection antibodies are typically in excess compared to the amount of antigen in the sample. Analyte-specific antibody (capture antibody) is pre-coated onto a microplate. The sample is added and any analyte present is bound by the immobilized antibody. An enzyme-linked analyte-specific detection antibody is added, that binds to a second epitope on the analyte forming the analyte-antibody complex. The substrate is added and converted by the enzyme, thereby producing a color product in proportion to the amount of analyte bound in the initial reaction (signal increases as analyte concentration increases). Substrate hydrolysis product is proportional to the amount of antibody present.

Quantitative ELISAs: ELISA results can be read as positive or negative (presence or absence of color change). However, the intensity of the color change is roughly proportional to the degree of positively of the sample: the more intense the color change, the greater the amount of Ag:Ab reaction occurring in the sample. However, because the

color change is dependent on the presence of an enzyme, small quantities of an enzyme can convert as much substrate as a large quantity of substrate, if given sufficient time.

1.21.2 Choice of assay

The selection of assay hinges on a number of factors, all of which must be considered before development begins.

- Is antibody or antigen to be measured?
- Is the assay to be quantitative or qualitative?
- Is a specific antibody class to be measured?
- What reagents are available?
- What is the avidity/affinity of available antibodies/monoclonal antibodies?

Optimization of assay conditions: The choice of assay conditions is an important aspect of ELISA design criteria. To determine the optimal conditions for an assay it is necessary to select suitable reagents, incubation times and temperatures. As a general rule, the higher the temperature, or the higher the concentration of reagents, the sooner equilibrium is reached. Increasing the temperature of incubation also decreases the assay sensitivity. Regardless of the incubation temperature used, the assay time should be sufficient to allow equilibrium to be reached. At room temperature, equilibrium is reached usually between 1-4 hours.

1.21.3 Methodology

Every non-competitive assay will involve six basic steps:

- The adsorption of antigen or antibody to the solid phase
- The addition of sample and subsequent reagents

- The incubation and washing stages
- The addition of enzyme-labeled antigen or antibody
- The addition of specific substrate
- The measurement and interpretation of results

1.21.4 The Components of the ELISA test

a) Assay plates

Disposable, flat-bottomed 96 well microtitre trays are used. These are manufactured by several companies in rigid polystyrene (Alpha, Becton-Dickinson, Dynatech, Flow, Nunc Sterilin) and flexible polyvinylchloride (PVC) (Becton-Dickinson, Dynatech, Flow). The polystyrene plates may be specially processed for use in ELISA (Flow Laboratories and Nunc). Dynatech also manufacture plates in Immulon plastic specifically for ELISA.

Polystyrene plates are normally more suitable than PVC because the binding capacity of polystyrene is greater for most antigens. Since the binding to plastic varies with the chemistry of the antigen, it is essential to test a variety of plates to find the most suitable for a specific antigen. Immobilization of antigen or antibody on the solid phase is probably as a result of hydrophobic interactions between molecules of the solid phase and the antigen or antibody. Solid-phase immunoassays based on immobilization of the solid-phase reactants are successful because the adsorptive process is hydrophobic and the subsequent immunochemical reactions with the immobilized reactant are largely hydrophilic (Maggio ET ,1980).

b) Solid phase reagent

The solid phases are used to coat the plate to capture the analyte of interest. In our work, the four solid phases that have been used are protein-G, protein-L, goat anti-mouse IgG and goat anti-mouse IgG Fc specific. Protein-G is obtained from the slime layer of the

bacteria Streptococcus sp, it has affinity towards the Fc portion of the IgG. Protein-L is obtained from the slime layer of the bacteria *Peptostreptococcus magnus*, has affinity towards the light chain of the IgG. Goat anti-mouse IgG is obtained from the goat by immunizing the goat with the mouse IgG has affinity towards the mouse IgG. Goat anti-mouse IgG Fc is obtained from the goat by immunizing the goat with mouse IgG Fc portion and it has affinity towards the mouse IgG Fc portion.

c) Buffers

A number of buffer systems are in use. Generally the antigen is coated in phosphate buffer saline 0.1M pH 7.2-7.4, although bicarbonate buffers of pH 8-9 are also described. There must be absolutely no detergent in this buffer. For subsequent washes and dilutions, phosphate buffered saline is used with the addition of blocking agent or detergent to reduce any further non-specific binding of reagents to the plate or previously bound reagents. Tween 20 at 0.05% is the most common, and bovine serum albumin (BSA) may be used on its own at 0.5% or in combination with Tween-20. The buffer used when adding substrate is chosen to maximize enzyme activity. When the enzyme is alkaline phosphatase, a sodium carbonate buffer 0.05 M, pH 9.8, with or without 0.001-M magnesium chloride, is used. An alternative is 0.1 M diethanolamine at the same pH. For peroxidase conjugates a citrate buffer 0.05M, pH 4, is often used for the substrate buffer. Sodium azide (0.02%) can be added as a preservative for buffers prior to the substrate buffer when the enzyme is alkaline phosphatase. It must not be used with peroxidase as it inhibits its activity.

d) Reporter Enzymes

i) Alkaline Phosphatase (APASE): Alkaline phosphatase is chemically orthophosphoric-*monoester phosphohydrolase*. It catalyzes the hydrolysis of an orthophosphoric

monoester to yield an alchol and an orthophosphate. It has an optimal activity at alkaline pH. The enzyme has a strict specificity requirement for the phosophoryl part of the substrate, but not for the nonphoshoryl part. It has wide substrate specificity. Commonly used colorimetric substrates include p-nitrophenyl phosphate, phenolphthalein monophosphate, and thymophthalein monophosphate and the wide spectra of fluorogenic substrates. All mammalian APASE are zinc metaloenzymes. The active site zinc ion is necessary for catalysis. The molecular weight of APASE varies from 84,000 to 150,000 Da depending on the source of enzyme (Fernley 1971). Use of PBS in the experiments with AP will cause 50% decrease in the signal generation (Kurstak.E 1985).

ii) Horseradish Peroxidase (HRPO): Peroxidase catalyzes the oxidation of a variety of compounds by hydrogen peroxide or related compounds. A general reaction mechanism of peroxidase involves an oxidation of the enzyme by hydrogen peroxide. HRPO is a holoenzyme with hematin (ferriprotoporphyrin IX) as the prosthetic group. HRPO is usually conjugated with a specific antibody. This conjugated antibody is called as the secondary antibody. In our work, the three conjugated enzymes were, goat anti-mouse IgG HRPO, protein-G peroxidase and protein-L peroxidase. Goat anti-mouse IgG HRPO has affinity towards the mouse IgG or mouse derived hybridomas. Protein-G peroxidase has affinity towards the Fc portion of the IgG. Protein-L peroxidase has affinity towards the Light chain of the IgG. These conjugates, when combined with the substrate TMB will generate a signal, whose intensity depends upon the concentration of the analyte bound.

e) Blocking agent and Detergent in ELISA

The high sensitivity of ELISA implies a stringent limitation to the acceptable background signal due to non-specifically bound reactants. Low background is usually achieved by thorough "blocking" of the test wells with an inert or irrelevant protein.

If a blocking agent is used together with detergent in ELISA, one must take into account that these reagents are competitors as far as blocking effect is concerned. Therefore they may counteract each other if not used with care. Blocking agents may be used in ELISA for blocking possible unoccupied solid surface after coating with one immuno-reactant to avoid non-specific immobilization of succeeding reactants. If detergent is present during incubation with secondary reactants, it might in some way interfere with the immunologic specificities or cause non-specific immobilization of the reactants (Esser 1990). If detergent is present during wash after secondary reactants, possible weak immunologic affinities might be broken by the washing activity of the detergent. Another reason for using a blocking agent would be to stabilize the immobilized reactant by steric support (Jitsukawa T. et al., 1989). This is relevant for storage or preservation of coated surfaces. A typical blocking agent would be a different macromolecule, large enough to establish a stable attachment to the surface, yet small enough to find its way between immuno- reactants, e.g. antibodies. Bovine serum albumin (BSA) of MW 67,000 is commonly used as a blocking agent. The problem of using both detergent and blocking agent occurs during the wash after 1st layer immobilization on the surface. If detergent is used in this wash, one may risk (depending on the detergent) unstable attachment of the succeeding blocking agent (Esser 1990). The optimal combination of blocking agent and

detergent seems to be achieved simply by omission of detergent until the wash after incubation with the last reactant.

Signal development

Signal development can be affected by several factors such as: type of substrate, incubation time, conjugate or substrate failure, wavelength and instruments.

High backgrounds

High backgrounds can be caused due to

- Insufficient blocking
- Altering incubation times
- Inadequate washing
- High concentrations of the reagents
- Contamination of components
- Contamination of plate during incubation

Reusing the plate sealers, reservoirs, or pipette tips may cause contamination.

Non-specific reactions between reagents and samples

Because of the complex nature of many of the samples and reagents, non-specific reactions are a frequent problem. A number of different methods have been employed to avoid this. Adding of wetting agents such as Tween 20 and Triton X100 to the diluent and washing buffers helps to reduce non-specific binding to the solid phase. It also significantly inhibits binding of the coating layer to the solid phase and is, therefore, not included in the coating buffer. The effect of adding Tween 20 to the wash fluid is to reduce the variability of results. Proteins are added in ELISAs to minimize non-specific interactions. Another cause of non-specific reactions is rheumatoid factor, which is an antiglobulin that recognizes Fc portions of immunoglobulins.

1.21.5 ELISA in serum and plasma

It is important to store sera at –20°C or lower before use and to avoid repeated freezing and thawing which reduces the antibody titre. Sera are usually tested at the dilution of 1/100-1/200. When estimating substances in serum or plasma, serum contents generally suppress the antigen-antibody reaction. This is called as the matrix phenomenon or effect and serum should be diluted to minimize the effect. In addition, serum contains antibody fraction reacting with allogenic antibody. This allogenic antibody reacting Ig (a kind of rheumatic factor) bridges the first and the second ELISA antibody that leads the non-specific reaction, which will effect the accuracy and validity of the assay results (Yasuhito A et al 1989).

1.21.6 Advantages of ELISA

The advantages of solid-phase immunometric assays have, perhaps, been best realized in ELISA (Engvall& Perlmann1971) where the use of plastic, with its low non-specific protein binding characteristics, permits high concentrations of reagents to be used. As labels, enzymes offer a potential for greater sensitivity, compared with radioisotopes, because of the inherent amplification of signal that they provide (one molecule of enzyme generates many molecules of product). This amplification can be further increased by using radiolabelled substrates (ultrasensitive enzymatic radioimmunoassays) (Harris et al., 1979; Hsu et al., 1980). Additional advantages of ELISA are:

1. It is very sensitive and may be made very specific
2. It does not depend on the biological function of the antibody

3. Large number of tests may be done simultaneously.

4. It uses small quantities of reagents and is therefore relatively cheap.

5. The substrate can be chosen according to the available instrumentation.

6. The flexibility of the test allows it to be adapted to almost any situation.

CHAPTER-II
Comparative Evaluation of the Lab Scale Bioreactors for Monoclonal antibody Production

2.1 Introduction

Monoclonal antibodies (Mabs) have wide applications as research tools, diagnostic tools, therapeutic agents such as "magic bullets"and immunosuppressives, radioimmuno imaging agents (for example tumors) and for neutralizing toxins such as bacterial toxins and venom. In all these applications, the primary methods available to generate research quantities of Mabs (10mg-100mg) are static tissue culture, spinner or roller systems and ascities fluid from mice. As demand for Mabs has increased, alternative *in-vitro* production methods have been developed.

There can be a number of problems with the traditional method of Mab production in ascities fluid that are alleviated by the use of these *in-vitro* systems. These problems and limitations include a) Mice injected with a hybridoma fail to form any tumor at all (Hendriksen and Leew, 1998). b) Complications involving the formation of solid tumors, in some mice. These examples highlight the ultimate lack of control that the researcher has over the process of ascities fluid production in mice (Hendriksen and Leew, 1998) and c) Contamination of the purified Mab with varying amounts of nonspecific antibodies when protein-G affinity purification is performed.

Animal ethics committees have pressed for a review (and in some instances banning) of the practice of ascities production in mice. The growing ethical concern

about Mab production by ascities induction along with the improvement in cell culture equipment and techniques have led to an increased emphasis on *in-vitro* methods. Also, Mabs produced by ascities induction sometimes have reduced antigen-binding activity compared to Mabs produced by the *in-vitro* method, presumably due to contamination with irrelevant murine IgG. Conventional low cell density culture methods permit *in-vitro* production of Mabs, which are released in the culture medium at concentrations between 1and 20μg and sometimes upto100 μg/mL (Falkenberg, 1998). The low concentration of Mabs in these cultures and the high degree of contamination with bovine serum proteins essential for the *in-vitro* culture of hybridoma cells could complicate purification methods. Contamination with bovine serum proteins, especially bovine IgG co-purifying in Protein G affinity purification can also limit the use of these Mabs in the field of clinical diagnosis and therapy, and in some basic research techniques that require Mabs of high purity. These have resulted in Bioreactors and Cell culture systems (*in-vitro* approaches) to provide an alternative to the ascities (*in-vivo* approach) production of monoclonal antibodies.

Bioreactors are often used in monoclonal antibody production as alternative systems to the ascities method for milligram to multigram quantities of yields. Worldwide interest in seeking alternative methods has resulted in the development of several *in-vitro* techniques.

There are four principal methods for *in-vitro* production of Mabs such as

1) Batch processing

2) Fed-batch processing

3) Continuous processing and

4) Perfusion processing.

With the advent of various commercially available *in-vitro* systems for production of highly concentrated Mab, in the past few years, effort has been invested in the design of high-density culture systems, leading to the development of various bioreactors. For example, hollow fiber systems can generate high yields of Mabs such as 100 mg/week on an average (Lowrey et al., 1994; Jackson et al., 1996; Kreutz et al., 1997). Autoclavable tumbling chamber systems (Jaspert et al., 1995), packed bed bioreactors in glass cylinders (Moro et al., 1994) offer alternative possibilities for growing multiple hybridomas simultaneously and producing large quantities of Mabs (25mg-1.5g/week). However, the risk of contamination of these bioreactors with infectious agents can be substantial, particularly if multiple manipulations are involved. In addition, such technology is typically limited to a few specialized laboratories with routine requirements for large quantities of Mabs since dedicated relatively expensive specialized equipment is needed.

In this study, our bioreactors and culture systems are based on the batch process and fed-batch process. We have extensively used these bioreactors employing several hybridomas as an alternative to mice for antibody production both for humane and practical reasons. Not every cell culture system that is marketed can be a good alternative to the ascities production. To recommend the best bioreactor or cell culture system for the laboratory use, it is essential to evaluate the various available bioreactors. Many existing *in-vitro* systems are technically demanding and have hidden expenses.

The most relevant type of *in-vitro* alternative depends largely on the quantity and purity of monoclonal antibody needed. There are, however, some criteria for

rating each system. The ideal *in-vitro* method should have a combination of the following features (Falkenberg, F.W. et al., 1993,95):

Table 2.1: Desired Features of an ideal Bioreactor

1.Should have expendable material cost similar to that of a mouse.
2.Should be a simple technique, requiring no special personnel expertise beyond that for typical Cell culture procedures.
3.Should not require prior adaptation of the hybridomas or special culture conditions.
4.Should have significantly higher concentration and quality of Mabs than seen in simple stationary cultures.
5.Should be a closed, reusable system with minimal risk of contamination.
6.Should be flexible to scaling up to some extent.
7.Should produce adequate quantities of Mabs in a reasonable period of time.
8.Should be reusable to decrease the capital investment costs.

Only few *in-vitro* methods can meet all these criteria. Thus bioreactors were evaluated based on these criteria for comparative efficiencies. In this study, we compared the relative yields of the same hybridoma secreting mouse monoclonal antibodies in different systems. Thus, the different methods employed and results obtained to produce monoclonal antibodies are described. To evaluate the production of monoclonal antibodies, four different media were used in a combination with four different bioreactors based on different principles of construction and operating mechanism.

Reusability of the bioreactors beyond the usage cycles that were recommended by the manufacturer was explored for cost-effective usage of the bioreactors in a research

setting. An innovative approach of perfluorocarbon supplementation for better oxygenation of the bioreactor was explored to evaluate its effect on Mab production yield.

2.1.1 Hypothesis and Objectives

Hypothesis

Ascities production of Mabs secreted by hybridomas has been the classical way and is continued even today. This is inspite of a) the relatively impure preparations of Mab obtained due to endogenous immunoglobulins and b) the animal welfare concerns of continued use of ascities method of production.

Several disposable lab scale bioreactors have been introduced as alternatives but have been cost prohibitive today. In my thesis, I speculated that atleast some of these bioreactors could be repeatedly used for several cycles, without compromising antibody yields and clogging bioreactors for cost effective Mab production. I also hypothesize that introduction of PFC as a component in the media for better oxygenation could be beneficial for antibody production.

Objectives

The objective for this set of experiments was to

- Evaluate the various lab scale bioreactors and compare the yields of Mabs
- Explore the various commercially available media in combination with various bioreactors for evaluation of yields.
- Recommend the best bioreactor and media for future lab scale Mab production

2.2 Materials and Methods

2.2.1 Materials

2.2.1.1 Cell lines

The following cell lines were used in this thesis work.

A4A1 (anti-VEE): Secretes mouse IgG monoclonal antibody against Venezuela equine encephalitis. Obtained from DRES-DND (Suffield). 11D2(anti-WEE): Secretes mouse IgG monoclonal antibody against Western equine encephalitis. Obtained from DRES-DND (Suffield). 5B4D6(anti-VEE): Secretes mouse IgG monoclonal antibody against Venezuela equine encephalitis. Obtained from DRES-DND (Suffield). P54 (anti-Biotin): Secretes mouse IgG monoclonal antibody against Biotin. P106 (anti-M13 x anti-AP): Secretes bispecific monoclonal antibody, with one arm specific against M13 and the other arm specific against Alkaline Phosphatase. H.B 191(anti-mouse NK cell): Secretes IgG monoclonal antibody against mouse Natural killer cells, obtained from Dr.Terry Allen lab, Pharmacology, University of Alberta. FMC-63 (anti-CD-19): Secretes IgG monoclonal antibody against CD-19, obtained from Dr.Terry Allen lab, Pharmacology, University of Alberta.

As a positive result of my study and during the course of my thesis work, several of my lab associates have successfully adapted the I-Mab bag system for Mab production. The following are the cell lines used by them. P93 (anti-M13) : Secretes IgG monoclonal antibody against biotin, developed by Dr MR Suresh Lab, used by Sujatha Guttikonda. B27 (anti-CA125): Secretes IgG monoclonal antibody against CA125 antigen, developed by Dr MR Suresh Lab, used by Dr Jimmy and Dr P. Srinath. P111

(anti-Helicobacter pylori): Secretes IgG monoclonal antibody against Helicobacter pylori, developed by Dr MR Suresh Lab, used by Eunna Lee. S5 (anti-LPS of E.Coli O157): Secretes IgM monoclonal antibody against LPS(lipopolysaccharide) of E.Coli O157, obtained from ATCC.

2.2.1.2 Bioreactors/Culture Systems

1) Dialysis membrane separated two-chamber roller bioreactor: This bioreactor is commercially available as the miniPERM bioreactor from Sartorius Canada. 2) Cellulose acetate membrane based two-chamber tissue culture flask: This bioreactor flask is commercially available as the CL-1000 from Becton-Dickinson, Canada. 3) Gas permeable bag: This bioreactor bag is commercially available as the I-Mab and TC bags from DCL PEI Canada and TC-Tech Sartorius, Missisauga, Ontario, Canada respectively. 4) Roller culture bottle: The roller culture bottles were obtained from Sarstedt Canada. 5) Tissue culture flasks: 50 cm^2 and 75 cm^2 tissue culture flasks were obtained from Sarstedt, Canada.

2.2.1.3 Media and reagents

Four media were used in the experiments and they are : 1) Serum free medium SFM Hyclone, Utah, USA. 2) Dulbecco's modified Eagle's medium (DMEM) Gibco BRL, Burlington, Canada. 3) Becton Dickinson Cell Mab BD medium, BD Biosciences, Toronto Canada and RPMI-1640, Gibco BRL Life Sciences, Burlington, Canada. 4) Fetal Bovine Serum (FBS) was obtained from Sigma Scientific St.Louis, U.S.A. All the media were supplemented with 1% of penicillin-streptomycin and glutamine (PSG), Gibco BRL Life Sciences, Burlington, Canada at a concentration of 2mM L-glutamine, 50 units/mL penicillin, 50μg/mL streptomycin. FC-40:

Perfluorotributylamine, $(C_4F_9)_3N$ is a Perfluorocarbon obtained from 3M Company U.S.A. Pluronic® F-68 : is a surfactant and emulsifier obtained from the Sigma Scientific St.Louis USA. Trypan blue: A cell dye used for Dye exclusion test to check the viability, obtained from Sigma Scientific, St Louis, USA. Lactate test reagent and standard lactate solutions were obtained from Sigma Scientific, St Louis, and USA as a part of the test kit to monitor lactate production during the bioreactor campaign. FACS tubes, Centrifuge tubes were obtained from Fisher Scientific, Canada. Goat anti Mouse IgG FITC was obtained from Sigma Scientific, St Louis, and USA.

2.2.2 Methods

2.2.2.1 FACS analysis-Antibody producing Cell- selection

The FACS analysis was performed to analyze the antibody secreting hybridoma cells and sorting to isolate the putative high secretors. The A4A1, 5B4D6 cell lines were made into unlabelled & labeled sets with FITC-GAM in two tubes for each set. The procedure was performed as follows. The contents of the culture flasks were transferred into 50mL tubes. The tubes were centrifuged at 120 xg for 7 minutes. The supernatant was aspirated and 50mL of RPMI was added and centrifuged at 120 xg for 7 minutes. The cells were then counted and suspended at a concentration of $4x10^6$ cells/mL in RPMI. A 100 μL aliquot of cell suspension was taken in a FACS tube and 900 μL of RPMI was added as the control for unlabelled cells. A 20 μL sample of GAM-FITC (1:16 dilution of the stock) was added to 4x 10^6cells/mL-cell density as the test labelled cells. This was transferred to a 75 cm^2 flask and incubated at 37 °C in CO_2 incubator for 30 minutes. In three separate 96-well plates, 100 μL/well of RPMI –20% was dispensed for cell sorting. The plates were wrapped in aluminum foil to avoid contamination during transport. Cells from the flasks in the incubator were centrifuged and resuspended into 0.5 mL RPMI-10 and transported at 0°C for FACS analysis.

2.2.2.2 Perfluorocarbon emulsion preparation

A 45mL of 10%w/v of Pluronic surfactant F-68 surfactant solution was prepared in distilled water. This solution was warmed in the water bath at 37°C in a waterbath. To the warm Pluronic F-68 solution (external phase), 5mL of the Perfluorocarbon FC-40 (internal phase) was added drop by drop and subjected to

vigorous mixing using a homogeniser at 4000 rpm. This step was continued for 20 minutes to obtain a uniform and stable emulsion. The resulting emulsion was sterilized by autoclaving at 121 °C for 20 minutes. The emulsion after the autoclaving appeared to separate into two phases. But apparently upon shaking the emulsion was reformed instantaneously. The final emulsion was a 10%v/v of the perfluorocarbon.

2.2.2.3 Trypan Blue Dye Exclusion cell counting

Since many cell types proliferate at different rates, it is useful to estimate the number of cells plated. This can be easily accomplished by determining cell number using a hemacytometer (figure2.1). A hemacytometer is a specially designed glass slide with a 0.1 mm^3 chamber and a counting grid. It is often useful to estimate the number of dead cells that are present in the culture supernatant along with the live cells. Dye exclusion viability assays are used for this purpose. Viable cells exclude certain dyes (e.g., Trypan Blue, erythrosin, or naphthalene black), whereas non-viable cells stain due to breakdown in their cell membranes. The following protocol describes the steps used for cell counting using the hemacytometer. The hemacytometer was cleaned with 70% ethanol. The glass coverslip was also washed with 70% ethanol and placed over the grooves and semi-silvered counting area. A 20 µL of cell suspension was collected by a micropipette and an equal volume of 0.08% trypan blue dye solution was added and mixed well in a microcentrifuge tube. Using the micropipette, the cell suspension mixture was transferred to the edge of the hemacytometer and allowed to spread evenly by capillary action. Using the 10x objective of a microscope, the grid lines in chamber were focused. The slide was adjusted by moving the field so that one corner of 16 square grid area surrounded by three grid lines was under focus. The area of this square is $1mm^2$.

Cells in the four corner grids were counted and averaged to obtain the number of cells (n). The unstained cells are viable and the dead cells are the blue colored cells.The concentration of the sample is expressed by the expression C=n/v , where C= cell concentration (cells/mL), n=number of cells counted, v= volume counted (mL). For a hemacytometer, the depth of the chamber is 0.1mm and the area counted is $1mm^2$. So the volume is 0.1mm x $1.0mm^2$=$0.1mm^3$ or10^{-4}mL. Hence C=n/10^{-4}mL or nx10^4/mL. The cell concentration is equal to this number multiplied by the total volume of cell suspension. The final equation for counting the cells is C=(x/4) X 2 X 10^4 X volume of the cell suspension in total (volume of the culture). Where x= total no:of cells in the 4 squares counted. The formula for calculating the % cell viability is %cell viability = { total viable cells(unstained)/total cells(stained plus Unstained)}X100.

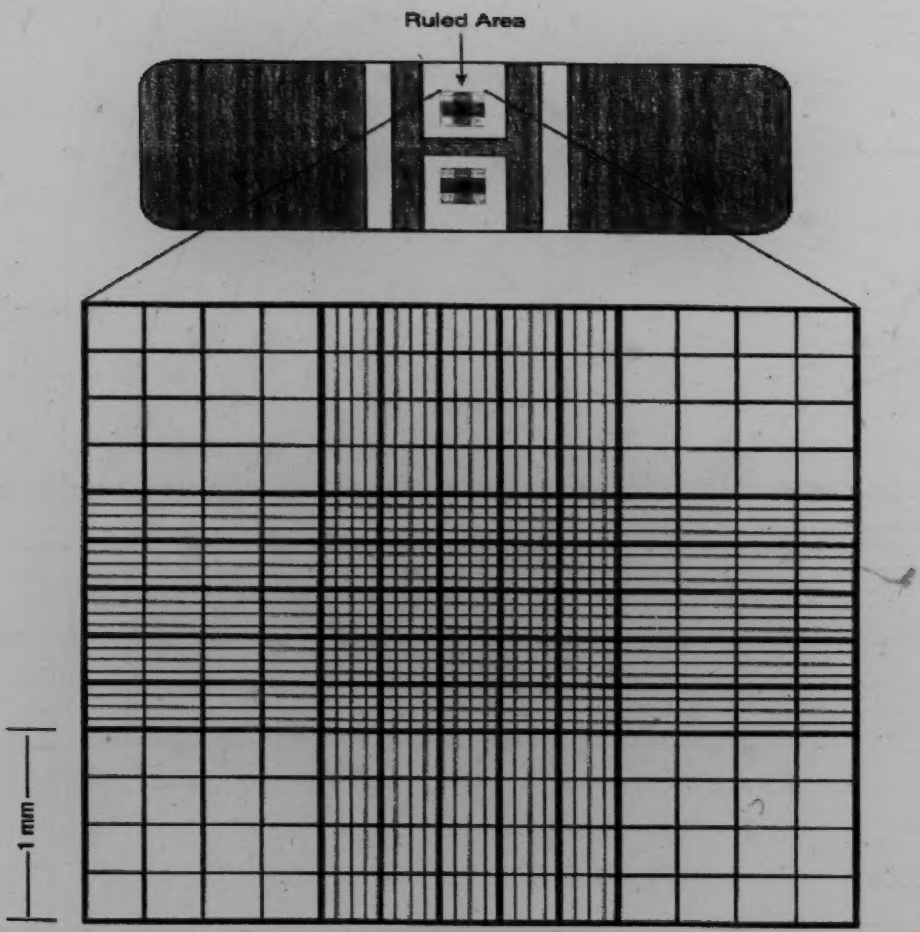


Figure2.1 Hemacytometer **(Reproduced from: Nunc brochure)**

2.2.2.4 BD medium Adaptation

The BD medium adaptation was a special weaning procedure (Becton Dickinson BD™ Cell Mab medium protocol) performed to adapt cells to this medium. The cells (A4A1) growing in the DMEM-10% medium were adapted in this stepwise adaptation process: 1) Cells were inoculated into medium consisting of 50% BD-10% medium and 50% of the DMEM-10% at a density of $2x10^5$ cells/mL in a $75cm^2$ flask. 2) The cells were passed into the medium combination of 75% BD-10% medium and finally

100 % with two passages in the same media combination before entering the next combination, to ensure that the cells were well adapted to the media combination. The cell viability was assessed with the trypan blue dye exclusion method. Between the media combination switches, cell viability of 100 % was observed.

2.2.2.5 Serum Free Medium adaptation-weaning method

The cell line A4A1 was adapted to the serum free medium (SFM) by the sequential serum deprivation of the cells growing in the DMEM 10% serum supplemented medium. Three 75-cm^2 flasks were set for the adaptation. Each flask had 10mL of the DMEM 10% serum supplemented medium. At this stage the adaptation was said to be 100 % serum supplemented, the cell viability was 100 %. To these three flasks, 9mL of DMEM-10% and 1mL of SFM was added to make it to 90%, this combination was allowed to grow for three days and the same combination of the media was replenished and allowed to grow for another passage of three days. The repeated incubation in the same combination of the media was carried over to ensure that the cells are compatible with the serum deprivation. A similar sequence in the decreasing % of serum supplemented medium and increasing % of SFM, followed by a incubation of 3 days and subsequent passage for three days in the same combination of the media was carried over until 0% serum supplementation and 100% SFM combination was reached.

2.2.2.6 Dialysis membrane separated two-chamber roller bioreactor

Construction: The miniPERM system (Figure 2.2) is a two-compartment bioreactor. The production compartment (40-mL in capacity) is separated from the nutrient module or compartment (550mL capacity) by a dialysis membrane with a molecular weight cut off of 12.5kD.

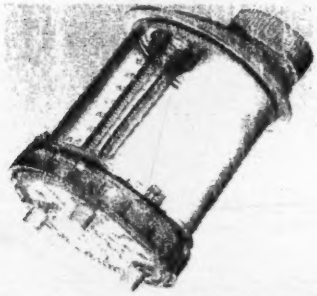


Figure 2.2 miniPERM bioreactor

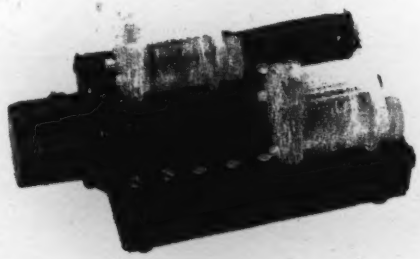


Figure 2.3 miniPERM on roller apparatus

(Reproduced from: VivaScience brochure)

Principle: When the production and nutrient modules are assembled, the membrane forms the dividing wall between the two compartments. Neither the cells, nor high molecular weight products released by the cells are able to pass through the membrane. However, nutrients and dissolved gases can diffuse from the nutrient module into the production module. At the same time, low molecular weight metabolites secreted by the cells can leave the production module *via* the membrane where they are diluted in the medium of the nutrient module. In addition, the nutrient module offers a large pH buffering capacity. One side of the production module is sealed with a thin, gas permeable silicone membrane, the other with a dialysis membrane.

Operation of modules: The cells to be cultivated are introduced into the production module, whereas the nutrient medium is filled into the nutrient module, which has a much larger volume. Neither the cells nor high molecular weight products released by the cells,

e.g. secreted monoclonal antibodies, can pass through the semipermeable dialysis membrane separating the two modules. Nutrients (glucose, amino acids), vitamins, ions and gases (O_2, CO_2) dissolved in the medium can, however, pass almost unhindered from the nutrient module into the production module. Because of more than 10-fold excess of nutrient medium, they provide the cells with the substances necessary for cultivation over a long period of time. At the same time, low molecular mass acidic (e.g.lactic acid), toxic (e.g. ammonium ions) and other products of cell metabolism diffuse from the production module through the dialysis membrane into the nutrient module where they are diluted and neutralized in a large excess of medium.

***Oxygen requirement and gas exchange*:** The oxygen consumption rate of hybridoma cells is in the order of 5µg O_2 per 10^6 cells per hour (Falkenberg 1998). This oxygen requirement is met by O_2 diffusing from the incubator atmosphere into the production module through the silicone rubber membrane. The CO_2 produced by the cells in corresponding quantities leaves the production module via the same route. Because of the high permeability of the silicone rubber membrane also to CO_2, the $NaHCO_3$ in the medium is in equilibrium with the CO_2 in the incubator atmosphere. Thus, to some extent, CO_2 removal *via* the silicone rubber membrane resembles its "exhalation" via a lung. The discharge of CO_2 from the culture vessel is further promoted by the fact that CO_2 dissolved in the culture medium, both physically and in the form of $NaHCO_3$, can pass through the dialysis membrane from the production module to the nutrient module. It can then be "exhaled" from the nutrient module through the silicone rubber membrane of the integrated pressure-equalizing module. Similarly, oxygen can reach the nutrient module from outside *via* the silicone rubber membrane of the pressure-equalizing module

where it is transferred into the nutrient medium. In the physically dissolved form it then passes through the dialysis membrane and reinforces the oxygen supply for the cells in the production module. Through these two silicone rubber membranes, the media in both modules are in contact with the incubator atmosphere.

High density cell culture: The design features of the miniPERM culture vessel make it possible to culture cells to considerably higher densities than in conventional culture procedures in which the oxygen and nutrient requirements of the cells can only be met by diffusion. Consequently, the cellular products will be secreted in much higher concentrations than is possible in conventional stationary tissue culture. Cells cultured in high density are, however, very much dependent on optimal culture conditions and much more sensitive to disturbances than is the case with cells conventionally cultivated in stationary culture at densities of 10^5 to a maximum of 10^6 cells/mL. Because of their high density, the cells are dependent on the continuous supply of large quantities of nutrients and oxygen and on the removal of metabolic waste products and CO_2. Optimal exchange rates between suspended cells and medium through the exchange membranes requires continuous circulation and mixing. Therefore, the cells must be kept in suspension at all times and must be agitated constantly and intensively. This is achieved by the following design features:

- The miniPERM bioreactor is rolled on a special turning device. This ensures that the cells are always kept in suspension and so guarantees optimal supply with nutrients and oxygen and removal of metabolic waste products and CO_2.
- The silicone rubber membrane of the production module is fitted with baffles. These take the form of nipples on the silicone rubber membrane which protrude into the

production modules so ensuring that the cells remain in suspension at all times and, at the same time, increasing the surface of the silicone rubber gas exchange membrane.

- The dialysis membrane is covered with a support grid on the side facing the nutrient module. This grid has two functions: 1) To protect the dialysis membrane from mechanical damage and 2) Provide a means of swirling the nutrient medium at the dialysis membrane surface thereby improving the diffusion of nutrients and metabolic products through the membrane.
- The pressure-equalizing module covered with a thin silicone rubber membrane and extending into the nutrient module also helps to mix the nutrient medium thoroughly while, at the same time, providing a means to exchange gases between the nutrient module and the incubator atmosphere.

Nutrient module feeding: The temperature of the nutrient medium should always be brought to 37°C before the module is filled. This measure is necessary for the following reason: After the nutrient module has been filled and closed and the miniPERM has been introduced into the incubator, the air above the medium in the nutrient module (about 150-200mL) will heat up and expand. The gas phase would increase in volume by about 24ml if the temperature in the space above the medium in the nutrient module would rise from 4°C to 37°C. This increase in volume would cause a substantial rise in pressure (more than 0.1bar) in the nutrient module of the miniPERM. There is, however, another effect, which is typical for cultures performed with $NaHCO_3$-buffered media in closed vessels and which causes a further rise of pressure in the nutrient module. When culture is started, there is usually atmospheric air rather than the CO_2-containing incubator atmosphere in the air space above the medium of the nutrient module. Therefore, the

$NaHCO_3$ in the medium decomposes, and CO_2 is released into the space above the medium of the nutrient module. The result is a further rise in pressure in the nutrient module and alkalization of the medium. These differences in the overall and CO_2 partial pressure will be leveled off by diffusion of gases through the thin silicone rubber membrane of the pressure equalizing module and CO_2 partial pressure and consequently, pH value will be readjusted to physiological levels within a few hours. At high cell densities, a moderate alkalization of the nutrient medium could even be advantageous for the culture since it would enlarge its absorbing and neutralizing capacity for acidic metabolic products of the cells.

Medium requirement and serum supplement: The cells should be cultured in the production module with a 35 mL medium supplemented with high concentration of 10% serum. In supply module on the other hand, the concentration of the serum supplement can be as lower as 5% with 450 mL media. The nutrient medium should be replaced as soon as there is a slight change in color from salmon-pink to a yellowish salmon-pink, as this signalizes that the medium is beginning to be depleted of nutrients. To monitor this, the lactate test was performed as cross check on the media utilization for periodic replinishment.

Handling procedure:

1. ***Preparation of cells for culture in the miniPERM bioreactor:*** The miniPERM culture was started with 35 mL of cell suspension containing $20x10^6$ cells in total. This seed cell culture is obtained from the hybridoma culture grown in a 75-cm^2 flask.

2. ***Preparation of the miniPERM culture vessel:*** The production modules are supplied sterile packed. The reusable nutrient module was sterilized by autoclaving before use.

- The nutrient module is assembled, packed in an autoclaving bag and autoclaved (121 °C, 30 min).
- The clear-peel was torn open in such a way that the production module resets on the caps of the Luer-Lock connectors.
- The nutrient module was gently clicked against the silicone rubber-sealing ring of the production module making the system leak proof. In doing so, the four snapper clips of the production module were snapped into the indentations on the rim of the nutrient module, pressing it against the silicone rubber sealing ring. This makes the assembled miniPERM ready for use.

3. ***Filling the miniPERM:*** The cell suspension was introduced into the production module with a syringe through one of the three ports equipped with Luer-Lock connectors. The miniPERM can be filled in the following way:

- miniPERM was mounted on a stand with the production module on top so that one of the three ports equipped with Luer-Lock connectors was positioned at the highest point.
- Two of the three caps were removed
- The prepared cell suspension(35mL) was drawn through a large gauge hypodermic needle about 100-150mm long
- The cell suspension was injected slowly into the production module with out the needle, but by screwing the syringe to the module's Leur-Lock.

4. ***Introducing the medium into the nutrient module:*** The nutrient module was filled with 350mL medium through the large neck opening, which is fitted with the screw cap. The miniPERM was placed on the three Luer-Lock connectors as the base for this operation. The miniPERM is then placed on the roller in incubator at 10 rpm. After incubation period of 1hour, the silicone rubber membrane of the production module will expand outwards as a result of the pressure generated in the gas phase of the nutrient module, and the silicone rubber membrane of the pressure equalizing tube will be compressed. To release the pressure generated in the nutrient module, the miniPERM was removed from the incubator after about 1hour and placed under the hood. The nutrient module cap was unscrewed about $3/4^{th}$ and the protruding production module pushed back gently and the cap tightened. By this manipulation, the pressure accumulated in the nutrient module will be released. The miniPERM was then kept back onto the roller apparatus (Figure 2.3) in the incubator.

5. ***Sampling and Cell harvesting***

Each time the production module is opened, both when samples are taken and when the culture was harvested, pressure accumulated in the nutrient module was released. The miniPERM was positioned on the stand so that the closed Luer-Lock connector of one of the ports of the production module was at the highest point (A) and the cap was removed at that connector. A 10mL syringe was screwed into the Luer-Lock and then followed by turning the miniPERM so that the elected sampling port with the attached syringe was positioned below the level of the liquid in the production compartment. The samples were drawn into the syringe. Before unscrewing the syringe, the miniPERM was turned so that the sampling port with the attached syringe again was at the uppermost

point. After removing the syringe, the Luer-Lock connector of the sampling port was closed with a new sterile cap and returned to the incubator. At every harvest, only 25 mL was drawn out and 10mL was left in the production module to serve as a preconditioned media for the next run. The harvested cell compartment volumes were centrifuged to remove cells and clarified supernatants were applied to a Protein G affinity column. The media change frequency in the nutrient module was 3-4 days. However, during the harvest, the nutrient module was also replenished.

This design satisfies all the prerequisites for high-density cell culture:

- Containment of cells and cell products (monoclonal antibodies) in a small cell culture and production module;
- A large excess of nutrient in a nutrient supply module;
- Efficient exchange of low molecular mass nutrients and metabolites between the production module and the supply module *via* a dialysis membrane;
- Efficient exchange of oxygen and carbon dioxide between the cell culture and the incubator atmosphere via silicone rubber membranes.

Lactate assay

A 1mL of the lactate reagent was taken in test tubes including the blank (Fresh media). To this 10 μL each of the three standard solutions (20mg/dL, 80mg/dL and 120mg/dL) provided in the test kit were added in the three respective tubes marked with the concentrations. Similarly 10μL of the test (sample) solutions were also added into the separate tubes designated as test. The tubes were incubated for 10 minutes.

Absorbance was recorded in a UV-spectrophotometer at 540nm for the blank, standard and test. A Lactate standard curve was obtained and the absorbance of test samples were extrapolated from the standard curve.

2.2.2.7 Cellulose acetate membrane based two chamber tissue culture flask (CL-1000)

Construction: This flask is a stationary high density cell culture system with a production chamber on the lower side with 15mL capacity, where cells are seeded. The production module is separated from the upper nutrient module by a 10-kDa molecular weight cut off (MWCO) cellulose acetate membrane. The nutrient module has a capacity of 1L volume.

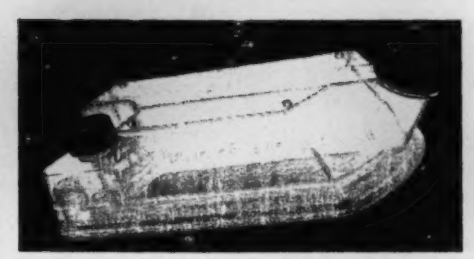


Figure 2.4 CL-1000

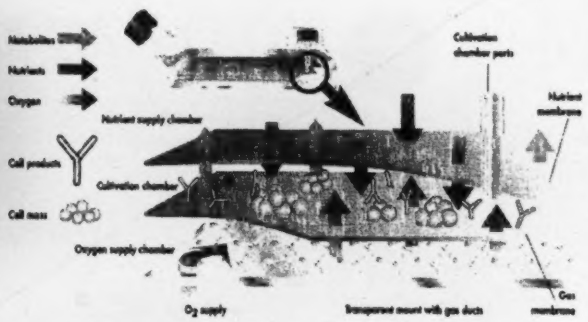


Figure 2.5 Mechanism of CL-1000

(Reproduced from: BD Biosciences brochure)

Principle: Cells are cultured in a cell compartment separated by an upper semipermeable membrane from the basal medium compartment. Nutrients and other small molecules pass across the semi-permeable membranes into and out of the cell compartment. Large molecules (>10, 000 MWCO) are retained within the cell compartment and need not be diluted by basal medium. The cells settle upon the bottom of the cell compartment on top of a gas exchange surface, across which oxygen and carbon dioxide rapidly diffuse. This

approach leads to high cell concentrations within the small cell compartment volume (Figure 2.4).

Role of the chambers: The cells to be cultivated are introduced into the lower cultivation chamber, whereas the nutrient medium is filled into the upper nutrient supply chamber, which has a much larger volume. Neither the cells nor high molecular weight products released by the cells, e.g. secreted monoclonal antibodies, can pass through the semipermeable cellulose acetate membrane separating the two chambers. Nutrients (glucose, amino acids), vitamins, ions and gases (O_2, CO_2) dissolved in the medium can, however, pass almost unhindered from the nutrient supply chamber into the cultivation

chamber. Because of ~60-100 fold excess of nutrient medium, they provide the cells with the substances necessary for cultivation over a long period of time. At the same time, low molecular mass acidic (e.g.lactic acid), toxic (e.g. ammonium ions) and other products of cell metabolism diffuse from the cultivation chamber through the cellulose acetate membrane into the nutrient module where they are diluted and neutralized in a large excess of medium (figure 2.5).

Handling procedure:

1.Preparation of cells for culture in the CL-1000

CL-1000 culture was started with 15 mL of cell suspension containing $30x10^6$cells in total. This seed cell culture is obtained from the static culture flask with 100 % viability from a full confluent 75-cm^2 flask.

2.Filling the CL-1000 nutrient chamber and cultivation chamber

The nutrient membrane must become wet before cells can be placed in the cell compartment. Hence 25 mL of the nutrient medium was placed into the nutrient compartment to wet the nutrient membrane.

With the nutrient chamber cap loosened, 15mL of cell preparation was inoculated into the cultivation chamber. It is very important to use a 10mL pipette for the cell inoculation. It is also important to remove trapped air bubbles and completely tighten the cap. The total cell number in this cell preparation was $2x10^6$ cells/mL. The medium in cultivation chamber was supplemented with 20% FBS and 1% PSG. The nutrient chamber was filled with 1000mL of the media supplemented with 10 % FBS and 1% PSG. The flask was then incubated in the CO_2 incubator for 2 weeks as recommended by the manufacturer.

3.Harvesting and changing the media

On the 15^{th} day of incubation, the nutrient medium compartment was emptied and filled with the fresh medium. The flask was harvested by removing 15 mL of the medium from the cultivation chamber, it was noticed that the volume of the medium has increased in the cultivation chamber by 5-8 mL. This was due to the osmotic flux into the cell compartment. Trypan blue dye exclusion test was done to estimate the cell count. Harvested cell suspension volumes were centrifuged at 20,000 X g for 20 minutes, the supernatant was collected and stored at –20 °C until purification. The cell pellet was discarded. The extra volume was left inside the chamber and the fresh cells with new medium and cell density make up was inoculated into the cultivation chamber. This was then let for another cycle of cultivation in the incubator.

2.2.2.8 Gas permeable bag

Construction: It is a gas permeable bag with a surface area of ~800 cm^2. The bag extends into one tube, which bifurcates at about 15cm from the bag into two tubes. Each of this tube has a roller clamp. These two tubes end up in two ports, one with a male port and the other with a female port. The female port has a luer lock end, which is designed to fit into the luer lock of the syringe, this is useful for the inoculation. The male port has a sliding cap, which can be used for harvesting. It also has two sampling sites for sampling the culture.

Figure 2.6 I-Mab bag (Reproduced from: DCL brochure)

Principle: The gas permeable bag is a monoclonal antibody production system (figure 2.6), based on the feature of high surface area/gas exchange, for free gas exchange of the CO_2/O_2 in a closed environment for increased cell growth, metabolism and decreased risk of contamination.

1.Handling/Inoculation procedure:

1. The roller clamp of the male port tubing was slided onto the tubing and closed. This roller clamp was not opened until the bag was harvested at the end of the experiment.
2. The bag was then stabilized on a ring stand at the lowest position possible by attaching a clamp to the non-flexible part of the base of one of the ports, with the ports and the tubing pointing up.
3. The white cap from the end of the tubing was removed and a 60cc syringe was attached firmly into the luer lock of the tubing. The syringe was then attached to the ring stand at the highest point as possible, so as to facilitate the medium and cells to flow into the bag by gravity.
4. The medium was then poured carefully, containing the hybridoma cells into the syringe and allowed to flow into the bag. The inoculation size was $50x10^6$ cells in total. Sometimes there would be air pockets trapped in the tubing. In order to facilitate the free flow, the tubing may be massaged. The mouth of the medium bottle should be wiped with a 70% alcohol swab before and after pouring each time to prevent contamination.
5. After all medium and cells have been added to the bag, the roller clamp was closed tightly. The only route and possibility of contamination could occur from the tubes, so in order to be more proactive against contamination, the tube between the roller clamp and the cap was filled with 95% alcohol.
6. The bag was incubated in a 37 °C incubator with 5% CO_2.

2.Harvesting the bag:

As per the manufacturer's protocol, the bag has to be incubated until the cell viability falls to 10-20%. This is usually reached at the mid 3rd week-4th week incubation period. It has frequently been noted that antibody production continues during the decline phase of growth and is often maximal during this phase (Emery, 1987;Birch,1987; Velez,1986); This is supported by the 2 theories - viable cells secrete antibodies and the dead cells release the antibodies (J.M.Renard et., al 1988).

1. After an incubation period of 3.5-4 weeks, The bag was removed from the incubator and hung on a ring stand with the ports facing downwards.
2. The ports were sprayed with 95% alcohol and the luer-lock cap of the female port was opened, the roller clamp of this port was loosened to facilitate the passage of culture harvest to flow freely through the port .
3. The culture supernatant was then collected into a sterile bottle and for cell density and further sample analysis, a quantity of 500 µL was kept aside. We explored the reusability of the bag and in this protocol, about 20 mL of the culture was left for the next run as a preconditioned media.
4. The collected supernatant was centrifuged at 20,000 X g for 20 minutes, the supernatant was then stored at –20°C until purified.
5. The bag was reused for several cycles for the same cell line, which is not the protocol recommended by the manufacturer. After some cycles, the roller clamps were broken, to replace this, pinch holders were substituted.

A gas permeable bag from a different manufacturer based on the same principle has been experimented, which had a one litre capacity, the construction is very simple and is self explanatory from its picture below Figure 2.7.

Figure 2.7 TC-Tech culture bags. (Reproduced from: TC-Tech web site)

2.2.2.9 Roller Culture Bottle

Construction: The roller culture bottle is made of polystyrene disposable plastic. The bottles vary in size and are usually with higher length to width ratio. The screw cap of the bottle is usually non vented (Figure 2.8)

Principle: When the cells are seeded into the round bottle that is then rolled around its long axis, the medium constantly bathes the cells on the walls of the bottle. If the cells are nonadherent, they will be agitated by the rolling action, but will remain in the medium. If the cells are adherent, they will gradually attach to the inner surface of the bottle and grow to form a monolayer. The increased ratio of the medium's surface area to its volume allows gas exchange to take place at an increased rate through the thin film of medium in contrast to the cells submerged in the deep part of the medium.

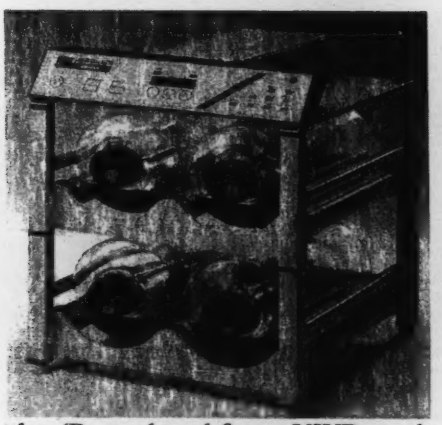


Figure 2.8 Roller Culture bottles (Reproduced from: VWR catalogue)

Handling Procedure:

1. The cells were seeded, with $50x10^6$ cells in total. The bottle was filled with 500mL of the medium.
2. Because the medium is CO_2/HCO_3 –buffered, the gas phase was purged with 5% CO_2. The CO_2 was blown into the bottle by pumping in the CO_2 gas through a pipette by bubbling it into the medium.
3. To facilitate enough CO_2 in the bottle, the bottle was bubbled with the CO_2, once every 3 days.
4. The bottle was harvested in the 4^{th} week. The culture supernatant was then collected into a sterile bottle and for cell density and further sample analysis, a quantity of 500 µL was kept aside. As a preconditioned media, about 20 mL of the culture was left for the next run.
5. The collected supernatant was centrifuged at 20,000 X g for 20 minutes, the supernatant was then stored at –20°C until purified.
6. The bottle was reused for subsequent cycles several times for the same cell line.

2.2.2.10 Tissue Culture Flask

Purpose of use: The reason behind using tissue culture flask in this evaluation process is to set a baseline control for the various *in-vitro* systems.

Handling Procedure:

1. The cell lines were grown in the 75 cm^2 flask until full confluency ($2x10^6$ cells/mL) was obtained, which took about 3-4 days.
2. The flask was harvested on day 3 or 4. As a preconditioned media, about 5mL of the culture was left for the next run with addition of 50mL of fresh media to the flask.
3. The supernatant collection process was continued until 500 mL of the supernatant was obtained.
4. The collected supernatants were centrifuged at 20,000 X g for 20 minutes, the supernatant was then stored at –20 °C until purified.

2.3 Results and Discussion

2.3.1 FACS analysis and sorting

The FACS analysis and sorting was performed as a cloning technique to identify and isolate the good Mab producing cells and avoid the non producing cells in the mixed population. The FACS sorting was performed to enhance the yields in the cell culture for higher quantities of Mabs from the cell culture. The FACS analysis data of the A4A1 cell line experiment is shown in the Figures 2.9 and 2.10. Figures in 2.9(a) are the negative control details of A4A1 cell line. Figure2.9 (a) shows the relative cell size on x-axis and granularity on the y-axis. Granularity increases with the cell death. Therefore the granularity is higher in the dead cells as the scale on the y-axis is moving up. The cells in gate A are the cells that are picked up for negative control background. This pattern is demonstrated in figure 2.9(b). The profile in figure 2.9(b) is FITC labeling on the x-axis in log scale and cell count in the linear scale on y-axis. Figures in 2.10 shows test details of A4A1 cell line FITC labeling. In Figure 2.10(a) the gate A shows the live cells that have been selected for cell sorting. Figure 2.10(b) shows the profile of the cells that have been labeled with GAMIgG-FITC. The profile in figure 2.10(b) shows the shift in the spectrum towards the right hand side towards the higher FITC labeling. The FACS computer sets up the channels B and D. Channel B explains the % of the cells above the spectrum of the unlabelled cells. The channel B % is reported to be 88.4 % in the sample information by the FACS machine. The channel D explains the % of the cells that are highly expressing the GAMIgG-FITC. The channel D % is reported to be 7.8% in the sample information by the FACS machine. The labeled cells were picked and sorted as one per well into the 96 well tissue culture plate.

FACS analysis report of the 5B4D6 cell line is shown in the figures 2.11a and b and 2.12 a and b. Figures in 2.11 are the negative control details of cell line 5B4D6. The figures in 2.12 shows the B channel is 47.1%, which represents the % of labelled cells with GAMIgG-FITC. The D channel is 4.9%, which represents the % of high expressing cells with the GAMIgG-FITC.

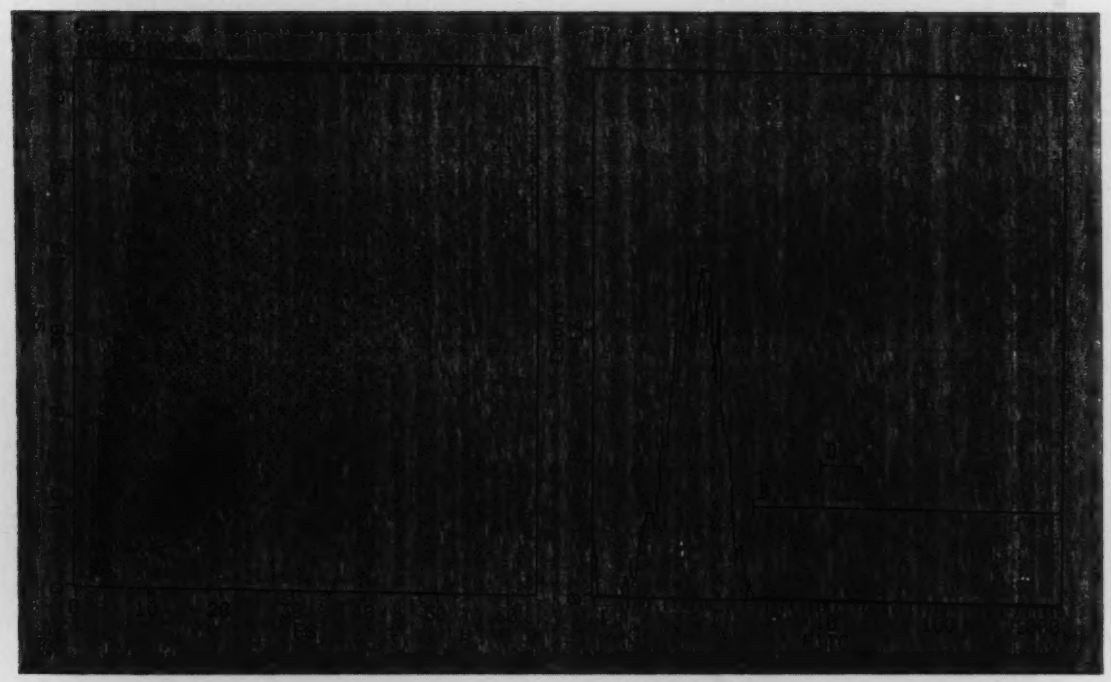


Figure 2.9 (a) A4A1 negative control Figure 2.9 (b)

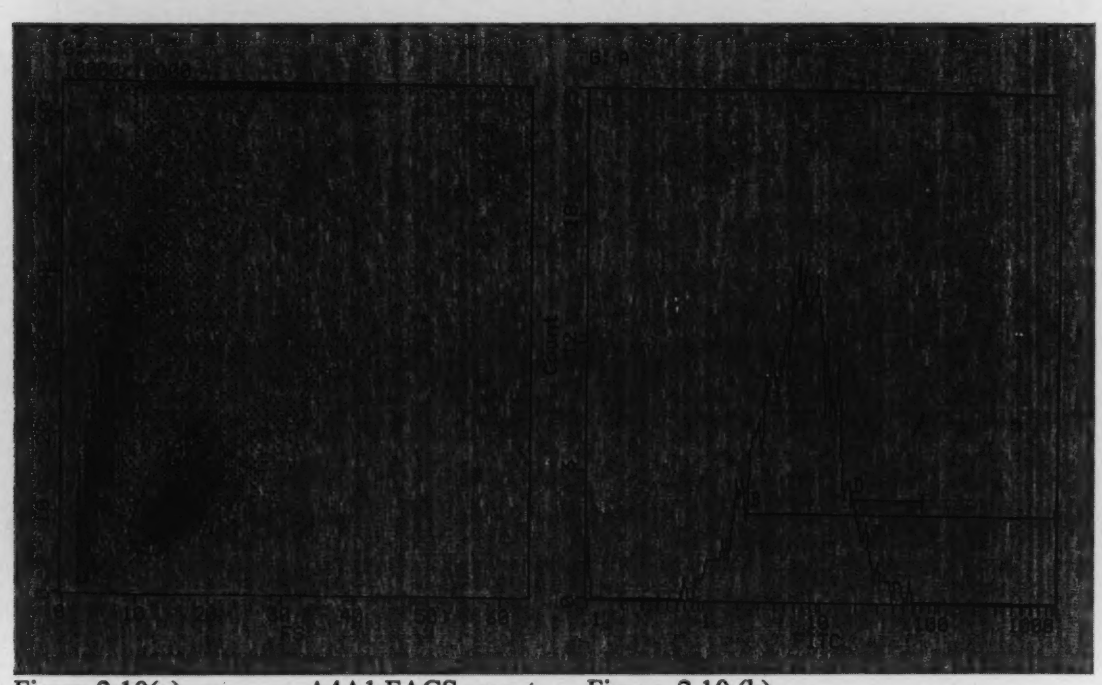


Figure 2.10(a) A4A1 FACS report Figure 2.10 (b)

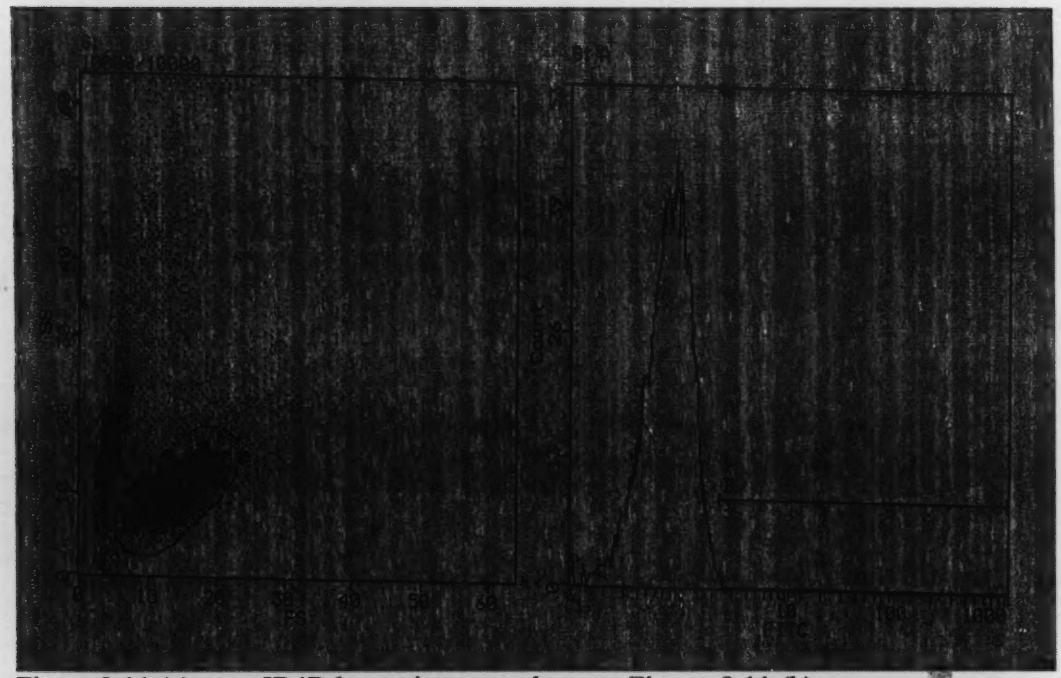


Figure 2.11 (a) 5B4D6 negative control Figure 2.11 (b)

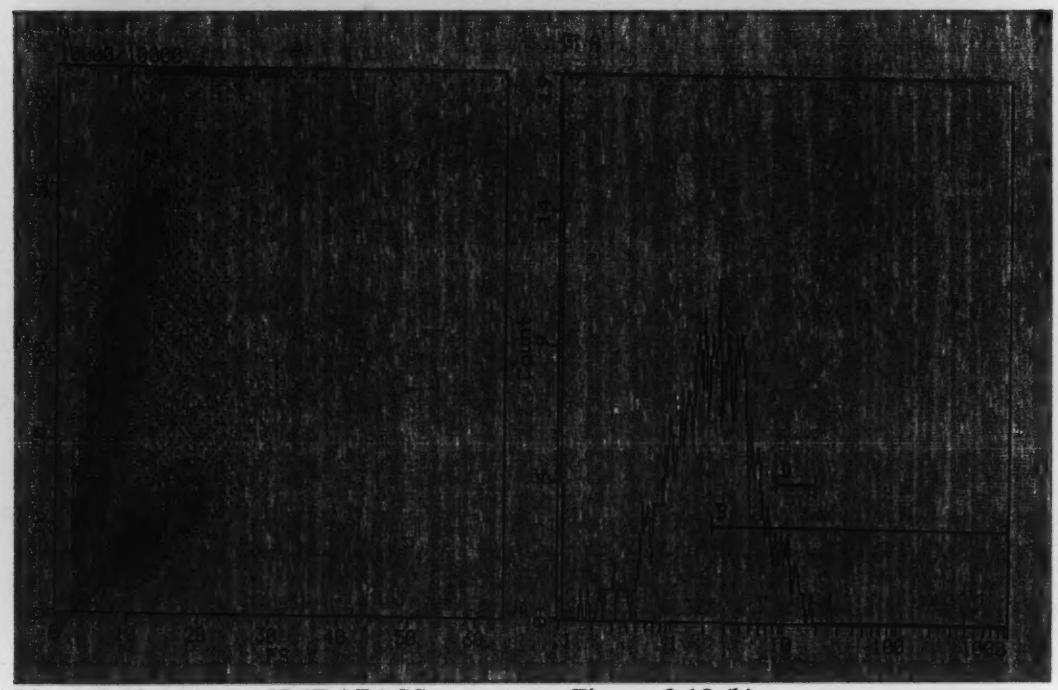


Figure 2.12 (a) 5B4D6 FACS report Figure 2.12 (b)

2.3.2 Comparison of the Mab yields in the various media

Factors affecting optimal Mab production include the type of medium employed and its level of serum supplementation. The composition of various commercial media vary. Hence the composition of medium have a profound effect on the Mab yields. A comparative representation of the Mab yields in the various production systems with various media is described here below. The yields are an average of two cycles of harvests for each system. Except for the stationary tissue culture flask with the yields being the representation of the supernatant accumulation upto 500mL. Four different media were tested with a single hybridoma cell line A4A1 to make a valid comparison in the different laboratory scale bioreactors.

2.3.3 Comparative yields of Mab production systems with RPMI medium

In RPMI, the order of the Mab yields were, I-Mab having the highest yield of 7.4±0.13mg/L followed by roller culture with 6.6mg/L which is closely trailed by CL-1000 with 6.1±0.0mg/L and subsequently tissue culture flask yield with 4.8mg/L. The miniPERM is on the lower side with approximately $1/10^{th}$ the tissue culture flask yield. In this comparative evaluation process it is obvious that the I-Mab bag is the superior hybridoma culture system for Mab production in RPMI medium ($p<0.05$ for a t-test between I-Mab and CL-1000). This is represented in figure 2.13.

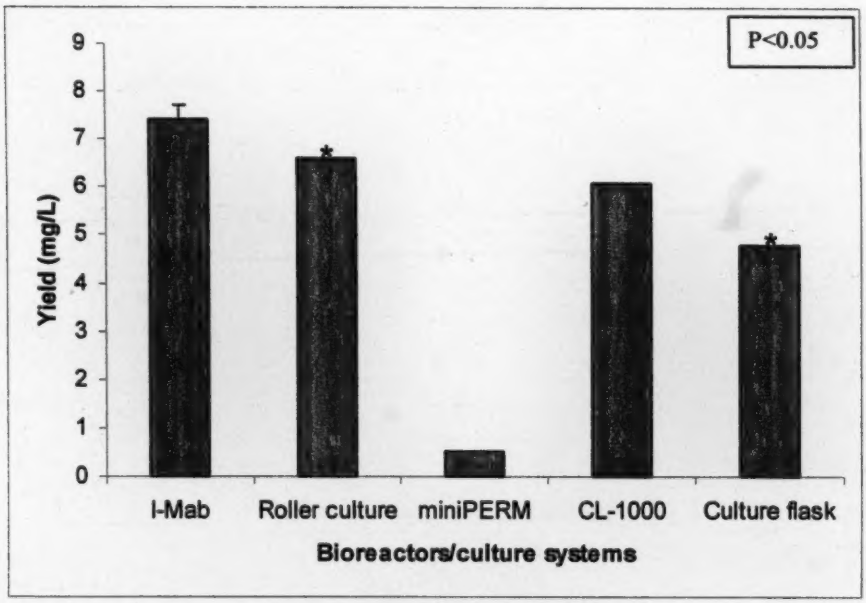


Figure 2.13: Comparative yields of A4A1 Mabs in RPMI media with various Bioreactors. (* Indicates the experiment with n=1).

2.3.4 Comparative yields of Mab production systems with DMEM

A comparative representation of the yields of Mab production in the various production systems in DMEM medium is represented in figure 2.14. DMEM has an higher concentration of glucose than that of RPMI medium, so it has more nutritional value than RPMI. Theoretically the yields obtained in the DMEM should be relatively higher than that of the RPMI medium. I-Mab bag yielded the highest amount of Mab with 13.9±0.63mg/L, and CL-1000 the lowest with 5.7±0.28mg/L ($p<0.05$ for a t-test between I-Mab and CL-1000). The yields of roller culture and the stationary culture had comparable yields of 11.2 and 10.4mg/L respectively. In all the culture systems harvests using DMEM with the exception of CL-1000 had approximately double the yield of the RPMI yields. From this, it is obvious that the I-Mab bag is again the best among the

various culture systems employed for Mab production with the combination of DMEM being better than that of the RPMI medium.

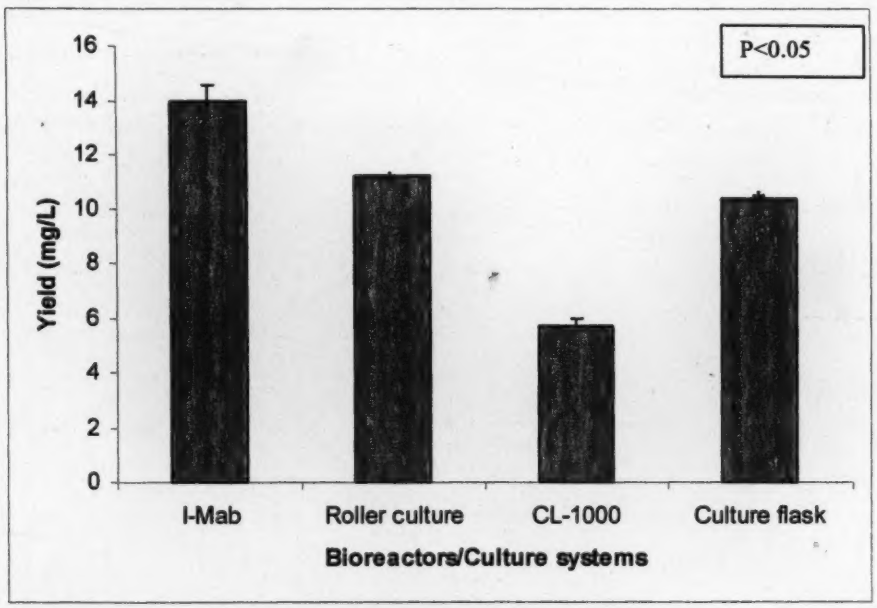


Figure 2.14: Comparative yields of A4A1 Mabs with DMEM medium in various bioreactors (* Indicates the experiments with n=1).

2.3.5 Comparative yields of Mab production systems with BD medium

The representation in Figure 2.15 is a comparison between the yields of Mab production in various production systems with BD medium. The BD medium is a special medium apparently designed for the hybridoma cell culture. The manufacturer does not disclose its composition and features. But it is evident that this is a better medium in terms of the yields in this study. Here again the I-Mab has resulted in the highest yield of 15.5±0.75mg/L. The roller culture and CL-1000 had the lowest yields of 7.6 and 6.3±0.33mg/L respectively. The yields of CL-1000 and roller culture were below that of the test control –tissue culture flask yield (10.9mg/L). In this analysis as well, it is

evident that the I-Mab bag is the best amongst the other culture systems for Mab production using BD medium ($p<0.05$ for a t-test between I-Mab and CL-1000).

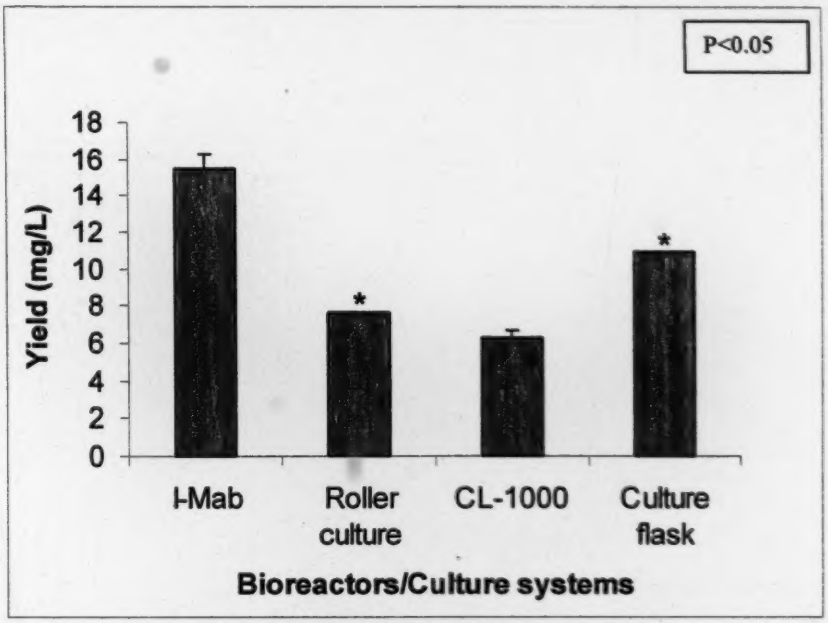


Figure 2.5: Comparitive yields of A4A1 Mabs in BD media with various bioreactors (* Indicates the experiments with n=1).

2.3.6 Comparative yields of Mab production systems with Serum free medium

Even though the serum free medium (SFM) needs a special adaptation of the cell line of interest to be cultivated, SFM has certain advantage in reducing the cultivation costs and downstream processing purification and purity of the Mab.The most striking observations under serum-free conditions were the relatively slow cell growth rates and the rapid formation of cellular domes or other morphological changes. Figure2.16 shows the yields of the I-Mab, roller culture and culture flask. The I-Mab has yielded 5.3±0.63mg/L, where as the roller culture and the culture flask were mere low of 1.7 and 1 mg/L respectively. I-Mab is concluded to be the superior culture system even in the SFM conditions. Trebak et al., 1999 have reported that the hybridoma cells produced

50% less antibody than the serum-supplemented cultures. Which is also observed in our studies and the yields here are even lower. Some authors (Frame and Hu, 1990; Oztruc and Palsson, 1990; Frame et al., 1989; Heath et al., 1989) reported the loss of Mab productivity during long-term culture of hybridomas adapted to SFM, due to the appearance of a non-producer cell population.

Sadettin et al., 1990 have reported in their Flow cytometric analysis that, there were two distinct cell populations with respect to intracellular and surface antibody concentrations. They also found that over time, the population shifted completely from high-producing cells to low-producing ones in response to the low serum environment. A fraction of the cells were lacking the antibody (non-producing cells), while the rest of the cells were producing (producing cells). Frame et al., 1989 and Heath et al., 1989 have reported that the loss in the observed antibody production rate was attributed to the increase in the size of non-producing cell population. This genetic instability is very important to the economics of long-term hybridoma cell culture (Dean, 1989). Frame et al., 1989 and Heath et al., 1989 have concluded that serum-containing factors prevent the loss of antibody productivity. We consistently observed a slower growth of the hybridoma in SFM compared with the serum-supplemented medium. R.P Singh et al., 1998 have reported that apoptosis accounts for most of the cell deaths that take place during the production of biopharmaceuticals from animal cell lines. In the past few years, the factors responsible for the induction of apoptosis in the bioreactor environment have been identified. A wide range of physiological and non-physiological factors, such as growth factor withdrawal, ionizing radiation and oxidative stress, have been found to result in the induction of apoptosis (Yang E et al., 1995). High levels of apoptosis have been reported

following deprivation of glucose, essential amino acids and to a lesser extent, non-essential amino acids (Mercille et al., 1994). In addition to these micromolecular nutrients, cell growth *in-vitro* is also dependent upon the presence of growth factors and cytokines, which are supplied in serum. Thus, in the absence of serum many cell lines, including those of industrial importance, will undergo apoptosis (RP Singh et al., 1998). A pressing observation was made and hypothesized that 1) appearance of a second clone in the culture, created by genetic drift has influenced the antibody production. 2) The stability of mRNA or the expression rate of the antibody-coding genes have been altered (RP Singh et al., 1998). This critical role of serum has not been considered during the development of serum/protein free media designed for the large-scale production of therapeutic proteins from animal cell lines. Alternatively, further improvements in productivity in SFM might be achieved by recloning the hybridomas in SFM using limiting dilution and selecting surviving producer clones (Moro et al., 1994). Recently, Liu et al., (1998) successfully generated murine hybridomas by performing the fusion process in SFM supplemented by interleukin-6 (IL-6). In terms of quality, the Mabs produced with the SFM display high purity, full activity, and integrity (Trebak et al 1999). Our experience differs with the procedure and results of M P.Bruce and co workers who have demonstrated that they have supplemented the SFM media with a small level of serum.

Decreased adventitious infectious agents and diseases such as the prions and bovine spongioform encephalopathy or madcow disease are eliminated with the use of serum free medium. Since serum free medium is defined for its components and is hence more likely to be accepted by regulatory bodies for therapeutic Mab

production.While the advantages of reducing or eliminating serum and other animal-derived products in medium used for manufacturing are obvious. The development of new medium formulations are complicated by various limitations. In general, cell lines have many nutritional requirements in common. However, each cell line also possesses its own requirements. Many cell types, although similar, may demonstrate different needs. Media developed for one line does not adequately meet the requirements of the other. Protocols employed for serum- supplemented cultures did not result in encouraging Mab yields when employed in serum-free conditions.

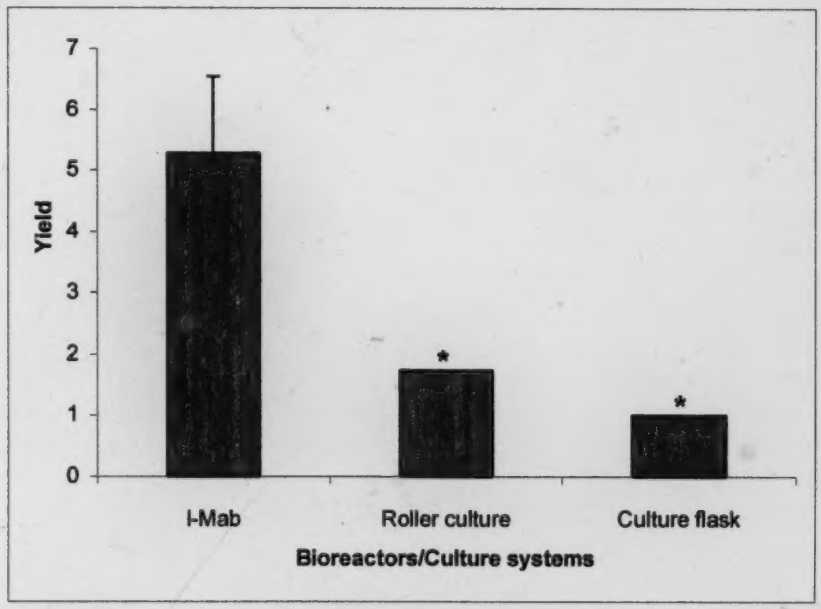


Figure 2.16: Comparative yields of A4A1 Mab in SFM media with various bioreactors (* Indicates the experiments with n=1).

2.3.7 Comparison between the various media in I-Mab bag

A comparison was made between the various media such as BD, RPMI, SFM and DMEM in the same I-Mab bag, with the same cell line A4A1 and the same culture conditions. Figure 2.17 shows the comparative yields with different media. It

appears that BD media is the best performing ($p<0.05$ for ANOVA test between media). But even though BD media resulted in good yields, it was not selected for the subsequent experiments because it is a special media in the market and very expensive when compared to the next best medium DMEM used in the evaluation process. So DMEM medium was used in the latter experiments as an alternative to BD medium. In DMEM, the cell line produces Mab well with the yields being significantly higher than the other media and slightly less than the BD media. The superiority of the BD media could not be explained as the manufacturer held the product constituents confidential. The yields in the DMEM are higher than that of the RPMI because the glucose concentration in DMEM is double that of the RPMI, resulting in more nutritional source to the cells. This could perhaps sustain cells for a relatively longer period for viability and Mab secretion.

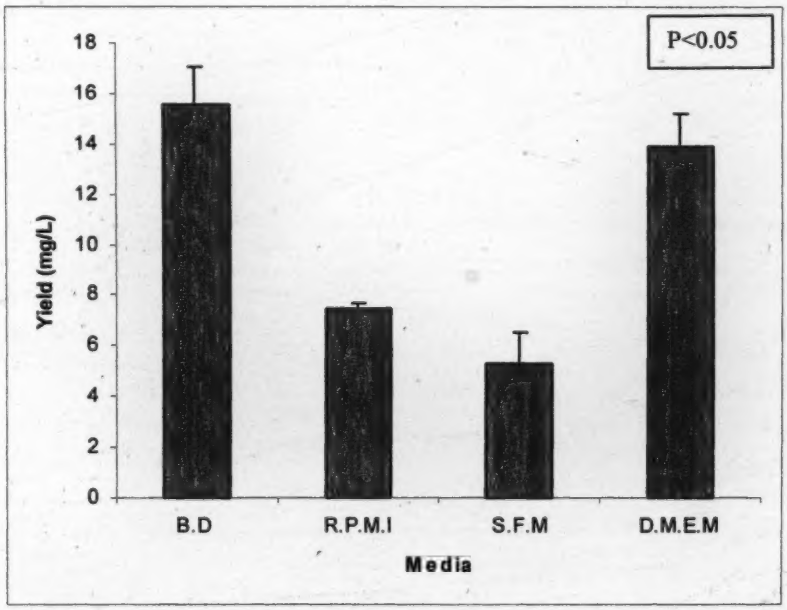


Figure 2.17: Comparison of the various media with yields of A4A1 Mab in I-Mab bag

2.3.8 Effect of perfluorocarbon supplement on the yields of I-Mab

In my study, the perfluorocarbon was used as a supplement to the medium in a concentration of 10% v/v (50 mL in 450mL of the I-Mab bag) as an oxygen transfer enhancer. A comparison was made between the yields of the I-Mab bag with and without the perfluorocarbons using the DMEM medium. Figure 2.18 shows the comparison of the yields of I-Mab with and without the perfluorocarbon supplement in the medium. The comparison showed that there is approximately a 40% increase in the production of the Mab with the perfluorocarbon supplementation and supports the previous results of Moo et al., 1998. The yield with perfluorocarbon, when compared to media without perfluorocarbon is significantly higher ($p<0.05$ for a t-test).

Perfluorochemicals (PFCs) are highly fluorinated, linear, cyclic or bicyclic organic chemically inert, fluorine-substituted hydrocarbons that can dissolve large volumes of non-polar gases. Oxygen solubility in PFC liquids is about 20-25 times greater than in either water or blood plasma under the same conditions. Unlike the chemical binding of O_2 to the porphyrin-iron sites of Hb, O_2 dissolution in PFC's is a simple passive process, in which gas molecules occupy so-called 'cavities' within the PFC liquid (KC.Lowe, et al., 1999). It has been suggested that PFCs may facilitate the transfer of oxygen into tissues by acting as "stepping stones" between red cells and blood vessel walls (K.C Lowe, et al., 1999). PFCs have enormous potential in cell biotechnology where, through their facilitation of gas supply, they stimulate cell division and biomass production, leading, in some instances, to increased production of valuable cellular products. PFCs are especially advantageous for use in cell and tissue culture systems, since they are heat

stable, readily sterilized (e.g. by autoclaving) and can be recovered from aqueous systems with the potential for recycling, thus making them economically viable. The oxygen transfer enhancement effect of perfluorocarbon emulsions was clearly demonstrated by the higher cell densities obtained in emulsion-supplemented systems (Lu et al., 1992).

A pressing need exists for better oxygen supply techniques in cell culture. For hybridoma cells consumption rates for oxygen are in the range of 5.0 ug/10^6 cells/hour (Miller et al., 1987; Wohlpart et al., 1990). At higher cell densities (above $20x10^6$ cells/mL), the oxygen supply becomes a limiting factor and accumulation of CO_2, lactic acid and other acidic metabolites leads to a decrease in pH below 7.0 and inhibition of cell proliferation (Falkenberg, 74th forum in immunology 1998). Sjorgren-Jansson and Jeanson, 1985 have identified that the possibility of cell viability could be improved by gassing with an oxygen/ CO_2 mixture when the cells became confluent. R.Pannell and C.Milstein 1991, have reported in their results that antibody production does not plateau even at the cell death stage. Researchers took advantage of PFCs high oxygen carrying capability and passed the oxygenated emulsions through the cultivation systems for supplying oxygen to cells. Lu et al., 1992, reported that the exponential-growth phase was prolonged, with a greater than sixfold increase in the population of viable cells in PFC emulsion supplemented systems. They suggested that the higher oxygen- transfer rates satisfied the increased oxygen demands of the greater cell populations.

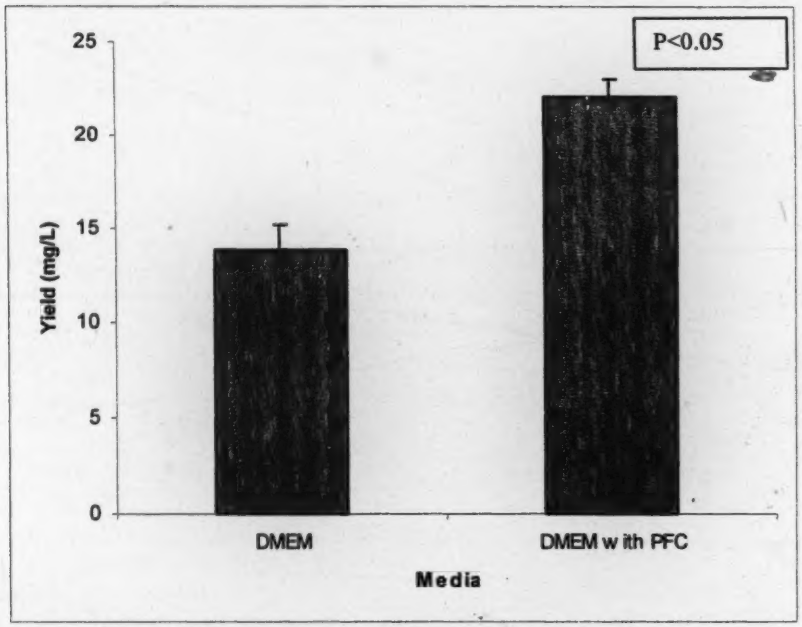


Figure 2.18: Comparison of A4A1 Mab yields with and without PFC in I-Mab bag

2.3.9 Reusability of I-Mab bag

In order to reduce the cost of Mab production, the bag was reused to find the possibility of reuse. I-Mab bag has been used for 10 repeated cycles of 1 month/cycle. Inspite of the repeated use of the bag, there was no significant deterioration in the efficiency of the bag performance. Figure 2.19 shows the yields in various cycles. The latter cycles that followed the SFM cycles have given good yields in the DMEM cycles and followed by the PFC-DMEM cycles. This indicates that the pores facilitating the gaseous exchange appear not to be compromised or clogged. This is in contrast with the miniPERM's production membrane, which appeared to get clogged creating pressure buildup between the two compartments (which was observed visually). The manufacturer has advertised I-Mab bag as a

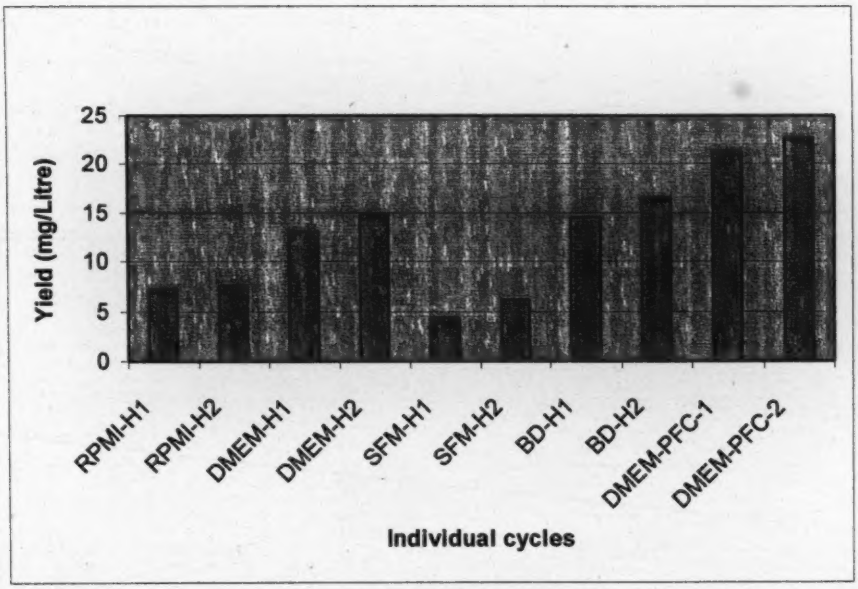


Figure 2.19: Reusability of the I-Mab bag, showing 10 repeated harvests

single use product, which in our hands has superceded the recommendations. The cost of the I-Mab bag obtained during my thesis was 120 Canadian dollars, which when used for 10 cycles of one month duration for each, was reduced to 12 dollars per run a month. Thus, with an approximate yield of 14mg/L, the cost of lab production was approximately $1.4/mg Mab (details in table 2.5). This when compared to the cost of mice, is very economical (see section 2.3.12), keeping aside factors such as cost and ethics of the maintenance of animal care. It is not only expensive to maintain the proper animal care facility with $0.4/day for each mice costing $8-10, but it is also very important to maintain the minimum standards of care and treatment. Facilities that violate the regulations of the 1985 Animal Welfare Act can face fines between $100 and a maximum of $2500 for each laboratory animal that is not treated according to the regulations. Currently, the policy states that "animals exhibiting signs of pain,

discomfort, or distress are expected to receive appropriate relief unless written scientific justification is provided in the animal activity proposal and approved by the Institutional animal care and use committees (IACUCs).

b) Downstream processing and purity: The down stream processing of Mabs from ascities to obtain the pure Mabs is very difficult, as the mouse IgG gets co-purified along with the Mabs. The contamination of the mouse IgG with Mabs would affect the efficiency of the Mab in its applications and its immunoreactivity. Besides this the individual batches of ascities may vary significantly in quality and quantity.

With the increasing restrictions, regulations and ethical committees associated with the care of laboratory animals and availability of comparatively cheaper *in-vitro* alternatives, the cost difference and feasibility is more favoring the use of non-animal methods for Mab production.

2.3.10 Comparative evaluation of bioreactor performance

Dialysis membrane separated two-chamber roller bioreactor

The hybridoma cells in the miniPERM bioreactor had given antibody yields that were well below the test control yields. The cells appeared to adhere to the silicon membrane, perhaps limiting the gaseous exchange in the production module. This system utilized the highest amount of medium (2940mL) for a month in comparison to the other bioreactor systems and gave the lowest yields (0.8mg/L). The features of this bioreactor were quiet innovative, but our experience and yields were not encouraging. For this reason, the miniPERM was not extensively studied and just confined to the studies only in the RPMI medium.

Cellulose acetate membrane based two-chamber tissue culture flask (CL-1000)

The CL-1000 flask has not given appreciable yields in the various media that were evaluated. In the case of DMEM, the yield was about 50% less than the test control (Figure 2.14). In the case of BD media the yield was about 60% less than the test control (Figure2.15). Even though the cells grew to a massive number, there was no clogging of the membrane and henceforth a good gaseous exchange from the bottom surface of the flask. The purpose of two-chamber system in the flask has served two purposes, -1) The cells were confined to a smaller volume and with good gaseous diffusion and 2) Downstream handling of the antibody supernatant was only 15-20 mL, unlike the I-Mab bag which yields 500mL to 1L for processing each time.

Gas permeable bag - I Mab bag

The yields in the various media were substantially higher than any other bioreactors in our evaluation ($p<0.05$). This is illustrated in Figure 2.17. This bioreactor has a very good gaseous exchange, whereby providing the environment for increased cellular viability. It also provides larger surface area for the cells to grow, thereby minimizing the contact inhibition and increasing the possibility of better cell metabolism yielding good antibody secretions. In our evaluation this was proven to be the technology for economic production of Mabs (section 2.3.12). In a research Beta site results (74^{th} Forum in Immunology), it indicates that the average yield from one 500 mL gas permeable bag was 36.9 mg, with a range of 2.0-102.0mg. Our yields have fallen in this range albeit lower than the average, and it should be noted that the yields are cell line specific and this cell line was not studied in the beta test analysis. Another type of gas

permeable bag TC-bag was assessed for productivity with the same cell line A4A1, but the yields when compared are inferior to the yields of the I-Mab. We speculated that the TC-bag gas diffusion is not perhaps as efficient as that of I-Mab bag. From the results of the coworkers (Dr Srinath), it is evident that the I-Mab bag yields are proportional to the volume of the bag from his experience with the cell line P54. In his case the yield was twice11.0mg (from 1L I-Mab bag) as that of my yield 6.6mg(from 500mL I-Mab bag).

Roller Culture Bottle

The yields in the roller culture bottle are appreciable, and were quiet close to the yields of I-Mab bag yields in RPMI and DMEM. But the yields are higher than tissue culture flask yields, in all the media except BD medium.

2.3.11 Antibody yields

The details of the A4A1 cell line used for bioreactor evaluation are given in the Table 2.2 The mean yield of the purified A4A1 Mab was 4.6mg±3.051, CV of 0.66 (n=31), with a range of 0.2-11.4mg of purified Mab.The cell lines as mentioned in the tables 2.3 and 2.4 were cultivated in the I-Mab bags as an outcome of the evaluation study of the cell line A4A1 in the various media with various systems. The cell lines that were studied include the monospecific and bispecific Mabs. The function of these, Mabs were described in detail in the materials section of this chapter.

2.3.12 Cost comparison

A cost comparison was performed to assess the cost for Mab production from various lab scale bioreactors, to give an idea of the cost factor involved with them. The details of this comparison are shown in table 2.5. For giving an overview cost

estimate of the ascities technique, the cost involved is as described here. Each mouse costs $10 and a maintenance cost of 40cents per day, so it costs $34 for 60 days. The cost of the labor would cost $75 at $25/hour for 2 months for 3 hours of priming and ascities collection. The priming material would cost $15 per mice. The average yield would sum up to 8mg (4 mg for tapping and 4 mg for terminal bleeding) for 2 months. The total cost of the antibody obtained from the ascities would cost $124.00 ($75+$34+$15). So the cost per mg would be $15.5. The cost/mg from the I-Mab ($14.08) is below the cost/mg that can be obtained from the I-Mab bioreactor bag. However, as the mouse cannot be reused, the feature of reusability in the case of I-Mab bioreactor bag (see section 2.3.9) is a cost attractive feature for the hybridoma labs.

Table 2.2 A comprehensive analysis of A4A1 Mab yields in the various bioreactors and media

System	Media	Harvest-Yield (mg)	Cell counts at harvest time	
			Live	Dead
miniPERM	RPMI	H1- 0.5	$93x10^6$	$10x10^6$
miniPERM	RPMI	H2- 0.5	$91x10^6$	$15x10^6$
miniPERM	RPMI	H3- 0.3	$93x10^6$	$18x10^6$
miniPERM	RPMI	H4- 0.5	$91x10^6$	$11x10^6$
miniPERM	RPMI	H5- 0.2	$92x10^6$	$21x10^6$
I-Mab	RPMI	H1-3.6	$21x10^6$	$151x10^6$
I-Mab	RPMI	H2- 3.8	$14x10^6$	$150x10^6$
RB	RPMI	H1-3.3	$18x10^6$	$143x10^6$
CL-1000	RPMI	H1-6.1	$84x10^6$	$189x10^6$
CL-1000	RPMI	H1-6.1	$87x10^6$	$188x10^6$
TC-flask	RPMI	Pool-2.4		
I-Mab	DMEM	H1-6.5	$31x10^6$	$250x10^6$
I-Mab	DMEM	H2-7.4	$36x10^6$	$263x10^6$
RB	DMEM	H1-5.6	$51x10^6$	$158x10^6$
CL-1000	DMEM	H2-5.5	$63x10^6$	$189x10^6$
CL-1000	DMEM	H2-5.9	$65x10^6$	$196x10^6$
TC-flask	DMEM	Pool-5.2		
I-Mab	BD	H1- 7.2	$34x10^6$	$262x10^6$
I-Mab	BD	H2- 8.3	$39x10^6$	$268x10^6$
RB	BD	H1- 3.8	$14x10^6$	$153x10^6$
CL-1000	BD	H1- 6.1	$78x10^6$	$193x10^6$
CL-1000	BD	H2- 6.6	$91x10^6$	$192x10^6$
TC-Flask	BD	Pool-5.5		
I-Mab	SFM	H1- 2.2	$2x10^6$	$89x10^6$
I-Mab	SFM	H2- 3.1	$3.5x10^6$	$89.5x10^6$
RB	SFM	H1- 0.9	$0.5x10^6$	$62.5x10^6$
TC-Flask	SFM	Pool- 0.5		
I-Mab	DMEM-PFC	H1- 10.7	$53x10^6$	$410x10^6$
I-Mab	DMEM-PFC	H2- 11.4	$51x10^6$	$444x10^6$

Table 2.3 A comprehensive analysis of the various cell lines that were cultivated in gas permeable bags.

Cell line	Media	Harvest-Yield (mg)	Cell count	
			Live	Dead
P54	DMEM	H1- 6.6	$37x10^6$	$125x10^6$
P54	DMEM	H2- 9.1	$39x10^6$	$150x10^6$
HB-191	DMEM	H1- 10.7	$77x10^6$	$265x10^6$
FMC-63	DMEM	H1- 8.2	$63x10^6$	$252x10^6$
P106	DMEM	H1- 44.7	$21x10^6$	$167x10^6$
11D2	DMEM	H1- 3.8	$11x10^6$	$201x10^6$
5B4D6	DMEM	H1- 7.4	$31x10^6$	$230x10^6$
A4A1 TC-Tech bag	DMEM	H1- 7.4	$33x10^6$	$281x10^6$
A4A1 TC-Tech bag	DMEM	H1- 7.0	$29x10^6$	$286x10^6$

Table 2.4: A collection of the data for various cell lines in I-Mab bag from the lab associates.

Cell line	System	Media	Harvest- Yield (mg)
P93	I-Mab	DMEM	H1- 5.9
P93	I-Mab	DMEM	H2- 6.9
P93	I-Mab	DMEM	H3- 6.5
P111	I-Mab(1L)	DMEM	7.9
S5	I-Mab(1L)	DMEM	17
P54	I-Mab(1L)	DMEM	11

Table 2.5: Cost comparison between the various lab scale bioreactors used in Mab production.

System	I-Mab	CL-1000	Roller Bottle	miniPERM	Tissue culture flask
Cost in $	120	350	20	550	10
Handling time in hours	1	2	4	10	3
Labor @ $25/hr	25	50	100	250	75
Medium consumption (DMEM)	1L	1.15L	1L	1L	1L
Medium expenses in $	20	20.3	20	20	20
Fetal bovine serum consumption	100mL	103mL	100mL	93.5mL	100mL
Serum expenses @ $30/mL	30	30.9	30	28.05	30
Yield/L in DMEM	13.9	5.7	11.2	0.8	5.2
Total cost	195	451.2	170	848.05	135
Cost/mg yield	14.03	79.16	15.18	1030.4	26.01
Reusability	Yes	Yes	Yes	No	Yes
Contamination risk	low	High	High	High	High

Note: Cost of Mab production from the ascities is $15.5 and details are provided in the section 2.3.12

2.4 Conclusion and Future work

For ethical reasons, strict rules and stringent laws were implemented around the world in recent years to minimize the use of ascites methods for producing monoclonal antibodies. More than 20 years ago, an *in-vitro* alternative-tissue culture was developed for production of monoclonal antibodies as an alternative to ascities (Mc Ardle 1997). Because of its simplicity and importance, this technique was widely adopted and used in every hybridoma labs around the globe.

For the last decade, researchers in Europe and the United States have systematically developed, validated, and adopted multiple *in-vitro* replacements to avoid the use of murine ascities. This innovation has progressed to the point that it is now possible to prohibit use of ascities. It replaces a method widely acknowledged to be cruel with more humane options, and promote a greater understanding and acceptance of the alternative approach to planning and conducting biomedical research.

From this evaluation of the bioreactors in a combination with the various media, it is evident that the gas permeable bags would be the ideal bioreactor system for the laboratory scale production of Mabs. The reusability of the I-Mab bags is another attractive feature for the antibody producers, which is a new feature and end point of my work. I-Mab bag is a cost effective alternative to ascities and existing *in-vitro* techniques. The I-Mab bag requires minimal laboratory requirements and hands-on time, coupled with the low start-up cost and the closed environment decreases the risk of contamination. The PFC media supplementation is a very promising approach for the enhanced production possibility for monoclonal antibody. Additionally, I demonstrated

approximately 40% increased yield in Mab production by increasing the oxygenation in the I-Mab bag with the PFC.

CHAPTER-III

A Generic Sandwich ELISA for the Estimation of Mouse Monoclonal antibodies

3.1 Introduction

Enzyme-Linked Immunosorbent Assay (ELISA) is a useful and powerful method in estimating μg/mL to pg/mL of analyte in solution, such as serum, urine and culture supernatants. Since their initial development in the early 1970s, noncompetitive solid-phase immunoassays (SPIAs) have gained widespread use in the measurment of both antigens and antibodies. They have supplanted or expanded many of the traditional techniques in diagnostic medicine and biological research. These procedures, which are simple to perform and give excellent results, take advantage of two important properties: the potentially very high specificity of antibodies for a given antigen and the extremely powerful amplification of chemical reactions achieved with enzymes, with colorimetric endpoints for easy estimation. All such procedures consist essentially of two steps, i.e., the immunological reaction to the desired antigen and the enzymatic indicator reaction to demonstrate the presence or absence of antibody-antigen or antibody –antibody reactions. A plethora of enzyme immunoassays have been reported, which are based on a variety of enzyme markers such as HRPO, AP, glucose oxidase, urease and others. In this work, to avoid the use of antigens, either due to their hazardous nature or the difficulty in procuring them and for economical considerations, we have explored the possibility of developing a generic SPIA , for detecting all mouse IgG class monoclonal antibodies. This approach is based on the popular sandwich assay design. In an attempt to build this

SPIA, we have evaluated several combinations of four solid phases and three solution phase enzyme conjugate reporters. This checkerboard format Figure3.1 allowed us to test twelve different sandwich SPIA formats for estimation of mouse IgGs. These formats were studied to select the best format of SPIA. This SPIA was explored for its ease of estimation of the raw culture supernatants as a measure of the secretory levels of the various hybridomas in the bioreactor for comparitive yields of murine Mabs.This format was explored for its applicability as a diagnostic tool for the murine antibody titres in the human serum, which can be used for either therapeutic or *in-vivo* diagnostic applications.

3.1.1 Hypothesis and Objectives

Hypothesis

In order to accurately estimate the mouse IgG from bioreactor harvests, a highly specific ELISA is needed. I hypothesize that a novel combination of immunological reagents specific only to mouse IgGs would estimate the secreted mouse IgG without cross reactivity to bovine IgG found in fetal bovine serum and chicken IgG found in the ovalbumin preparations.

Objectives

- Evaluation of the various ELISA formats obtained by combination of the various immuno-reagents.
- Identify an ELISA format that can measure mouse IgG which has no interference with the bovine IgG and chicken IgG
- Apply the ELISA format for the estimation of mouse IgG monoclonals in bioreactor culture supernatants

Solid Phase Capture reagent	Antibody or Protein conjugated solution phase tracers		
	Protein-G peroxidase	**Protein-L** Peroxidase	**Goat α mouse IgG** peroxidase
Goat α mouse IgG	**Goat α mouse IgG + Protein-G** peroxidase	**Goat α mouse IgG + Protein-L** peroxidase	**Goat α mouse IgG + Goat α mouse IgG** peroxidase
Goat α mouse IgG Fc	**Goat α mouse IgG Fc + Protein-G** peroxidase	**Goat α mouse IgG Fc + Protein-L** peroxidase	**Goat α mouse IgG Fc + Goat α mouse IgG** peroxidase
Protein-G	**Protein-G + Protein-G** peroxidase	**Protein-G + Protein-L** peroxidase	**Protein-G + Goat α mouse IgG** peroxidase
Protein-L	**Protein-L + Protein-G** peroxidase	**Protein-L + Protein-L** peroxidase	**Protein-L + Goat α mouse IgG** peroxidase

Figure 3.1 Format of various sandwich assays for estimation of mouse IgGs.

3.2 Materials and Methods

3.2.1 Materials

Immunoreagents : *The Solid Phase reagents:* Goat anti-mouse IgG, Goat anti-mouse IgG Fc specific, Protein-G and Protein-L and *Secondary antibody conjugates:* Goat anti-mouse IgG HRPO, Protein-L peroxidase, and Protein-G peroxidase were obtained from Sigma Chemical Co, St. Louis, U.S.A.

Phosphate buffer saline: NaH_2PO4 (anhydrous), Na_2HPO4 (anhydrous) and NaCl used for preparing the buffer was obtained from BDH chemicals, U.S.A.

Blocking solution: 1% Bovine serum albumin (Sigma scientific St. Louis U.S.A) in PBS, 1% Ovalbumin (Dr.Jeong Sim Lab, Agriculture and Forestry, UofA) in PBS.

TMB substrate solutions: KPL Inc, U.S.A.

Tween-80: Fisher Scientific, Canada.

Pipettes: Socorex U.S.A

ELISA plates: NUNC, Canada

ELISA plate reader: Molecular Devices Corporation U.S.A

Human serum: Obtained from Canadian Blood Services with the assistance of Christine Lutsiak(Ph.D student of Dr John Samuel Faculty of Pharmacy, University of Alberta).

3.2.2 Methods

3.2.2.1 Solid–Phase immuno-reagent plate coating and blocking

For all the 12 formats, the coating concentration for solid phase was 1 μg/mL. All the solid phase reagents were diluted in 1X PBS pH 7.2. The coating volume was 100 μL. The coated plates were incubated in the refrigerator for overnight at 2- 8°C. The plates were washed 3 times with PBS 200 μL for each wash and then tapped dry. This plate was then blocked with 200 μL of 1% BSA in 1X PBS for 2 hours at room temperature. This was followed by washing with 200 μL of PBS for each wash three times. The blocking was carried out to make the unbound or free polystyrene surfaces in the plate to be occupied by the albumin protein in the blocking solution. The washings were performed between steps of the assay to ensure that the unbound reagents (proteins, analytes, conjugate enzymes or antibodies) to be washed away and avoid the nonspecific binding and interference and also avoid high back grounds in the subsequent steps.

3.2.2.2 Analyte incubation

The analyte- mouse IgG was added to the plate in a doubling dilution starting at approximately 60 μg/mL and ending as low as 0.1 μg/mL. All the dilutions were performed in 1X PBS pH 7.2. The negative control or the blank reference was 1X PBS.The volume of the analyte was 100μL. The plate was incubated for 2 hours at room temperature with the plate sealer and gentle shaking on the shaker.The plate was then washed three times with 200 μL of the PBS for each wash and tapped dry.

3.2.2.3 Incubation of tracer antibody-conjugated peroxidase or protein L/G conjugated peroxidase

The antibody-conjugated or signal generating reporter enzymes were diluted in 1% BSA and the titre or the dilution of these were 1:10,000 for goat anti-mouse IgG HRPO, 1:2000 for protein-L and protein-G peroxidases. To the appropriate plate wells, 100 μL of the respective reporter enzymes were added according to the format that was designed. The plates were then incubated at room temperature with the plate sealer on the plate shaker for 1 hour. The plate was then washed three times with 1X PBS with Tween-20-0.01%, 200 μL for each wash and tapped dry.

3.2.2.4 Signal generation with peroxidase substrate

The peroxidase substrate was prepared by mixing equal volumes of the Solution A and Solution B and they were brought to the room temperature. These solutions are the components of the TMB substrate kit. The ELISA plate reader was set ready to read the plate at 650nm and the substrate solution was added to the plate for color development and intensity of the signal. The plate was read at different times starting at 10 min and increments of 10 minutes.

3.2.2.5 Detection of the antibody secretion levels in the raw cell culture supernatants

After a lengthy evaluation of the twelve formats, based on the results and taking into consideration the sensitivity issues and non-interference with the extraneous proteins in the blocking agents, the goat anti-mouse IgG Fc specific as solid phase and the goat anti-mouse IgG-HRPO was chosen to be the ideal format. This format was explored into its

applicability and its implication in the quantitation of the mouse hybridoma antibody secretion levels in the raw culture supernatants. The raw culture supernatants of the various bioreactor harvests (explained in the 2nd chapter) have been evaluated in this format. The ELISA design of this format in this experiment is the same as described earlier, except that the mouse IgG has been replaced with the raw culture supernatant as the analyte. The analytes were diluted 10 times and the subsequent calculations were multiplied by a factor of 10.

3.2.2.6 Spike and recovery of the mouse IgG in Human Serum

To simulate the study of the pharmacokinetics of the therapeutic Mabs, a measure of the amounts of Mab in the body at dosing intervals should be performed. The goat anti-mouse IgG Fc specific solid phase and goat anti-mouse IgG HRPO ELISA format (optimized) was used to quantify the serum levels of the Mab. This is a simulation experiment, mimicking the antibody in human blood. In this experiment, the mouse IgG (analyte) was diluted in human serum and the blocking of the plate, the goat anti-mouse IgG HRPO dilution was also performed with the human serum. A parallel quantification of the mouse IgG was performed with the similar conditions, except that the bovine serum albumin was used instead of the human serum.

3.3 Results and Discussion

3.3.1 Initial screening of the 12 different formats

Solid phase sandwich assays are the most versatile of immunoassay techniques that are explored in cancer, infectious diseases and other areas to detect or measure large molecular weight antigens. The principle is based on the formation of a ternary complex of an antigen or analyte sandwiched between two antibodies. The same strategy has been extended to solid phase assays forming binary or ternary complexes with other proteins as well. In this chapter, we investigated several such ternary complexes to estimate mouse IgG to monitor their production in bioreactors. Which was described in the chapter 2.

When all the 12 sandwich ELISA formats (Figure3.1) were evaluated, the formats that were successful with good linearity and sensitivity are those in the (Figure 3.2), with no shading. The formats that are shaded are the ones that lacked the linearity or showed plateauing with less signal and/or lack of signal development. Each of these assays were evaluated in two ranges of the mouse IgG as the analyte. The first was a higher range of mouse IgG from 0-60 μg/mL and a lower range of 0-1 μg/mL.

The 8 formats which utilized either protein-G peroxidase or protein-L peroxidase as tracers failed to generate a good signal with an expected linear response. (Figures 3.4, 3.6, 3.8, 3.10).

Protein-L solid phase with Protein-L peroxidase as the reporter enzyme and Protein-G solid phase with Protein-G peroxidase as the reporter enzyme were theorotically not expected to generate a response and were verified experimentally (Figures 3.10 b and 3.8 a).

Solid Phase Capture reagent	Antibody or Protein conjugated solution phase tracers		
	Protein-G peroxidase	**Protein-L** Peroxidase	**Goat α mouse IgG** peroxidase
Goat α mouse IgG	**Goat α mouse IgG + Protein-G** peroxidase	**Goat α mouse IgG + Protein-L** peroxidase	**Goat α mouse IgG + Goat α mouse IgG** peroxidase
Goat α mouse IgG Fc	**Goat α mouse IgG Fc + Protein-G** peroxidase	**Goat α mouse IgG Fc + Protein-L** peroxidase	**Goat α mouse IgG Fc + Goat α mouse IgG** peroxidase
Protein-G	**Protein-G + Protein-G** peroxidase	**Protein-G + Protein-L** peroxidase	**Protein-G + Goat α mouse IgG** peroxidase
Protein-L	**Protein-L + Protein-G** peroxidase	**Protein-L + Protein-L** peroxidase	**Protein-L + Goat α mouse IgG** peroxidase

Figure 3.2 Format of various sandwich assays for estimation of mouse IgG: The unsuccessful formats are shaded ,leaving further investigation of 4 formats in the last column.

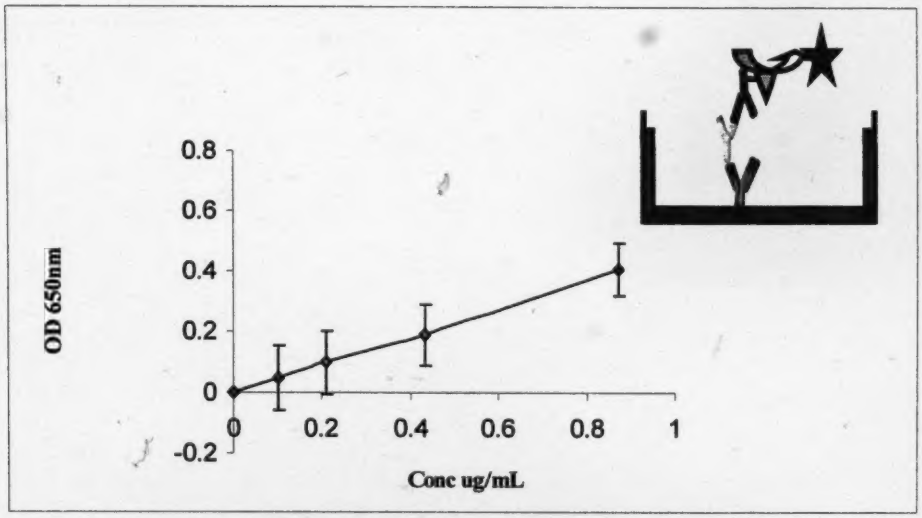


Figure 3.3 a: Goat anti-mouse IgG Fc as the solid phase and reporter enzyme goat anti-mouse IgG HRPO 1:10,000

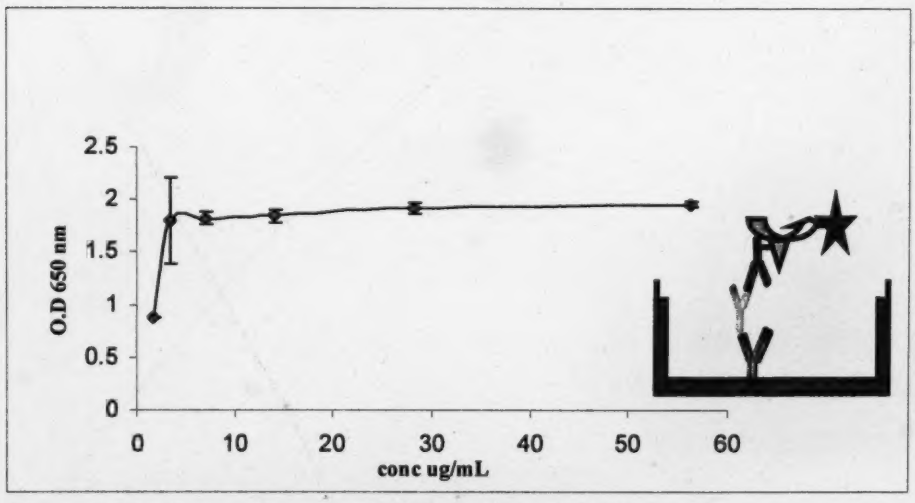


Figure 3.3 b: Goat anti-mouse IgG Fc as the solid phase and reporter enzyme goat anti-mouse IgG HRPO 1:10,000

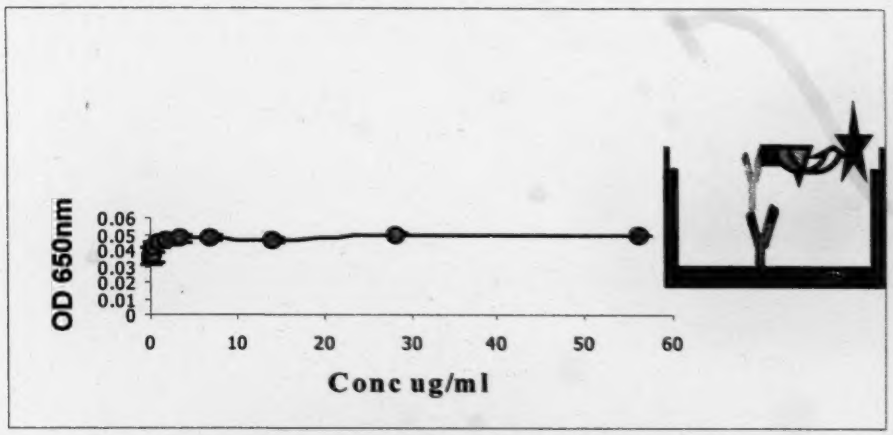


Figure 3.4 a: Goat anti-mouse IgG Fc specific on the solid phase and protein-L peroxidase as reporter enzyme 1:2000

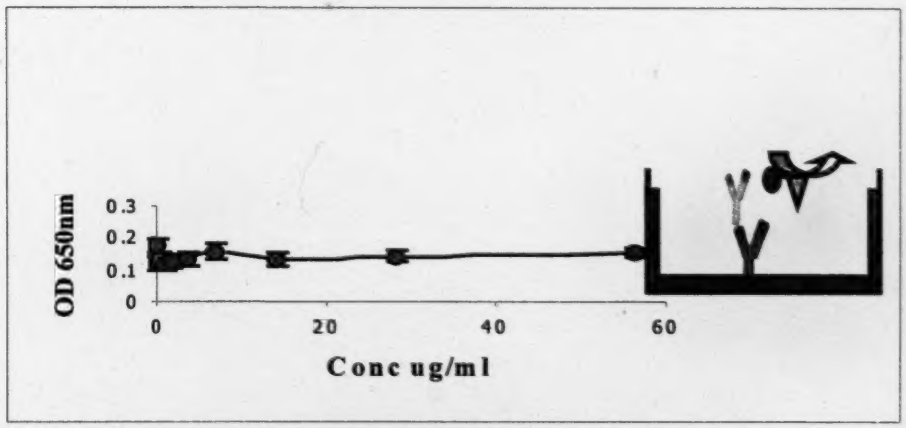


Figure 3.4 b: Goat anti-mouse IgG Fc specific on the solid phase and protein-G peroxidase as reporter enzyme 1:2000

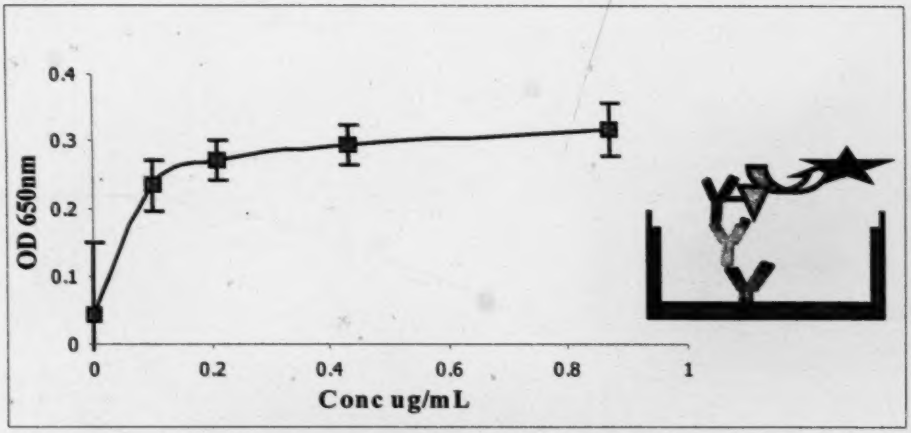


Figure 3.5 a: Goat anti-mouse on solid phase and goat anti-mouse IgG peroxidase as the reporter enzyme at 1:10,000

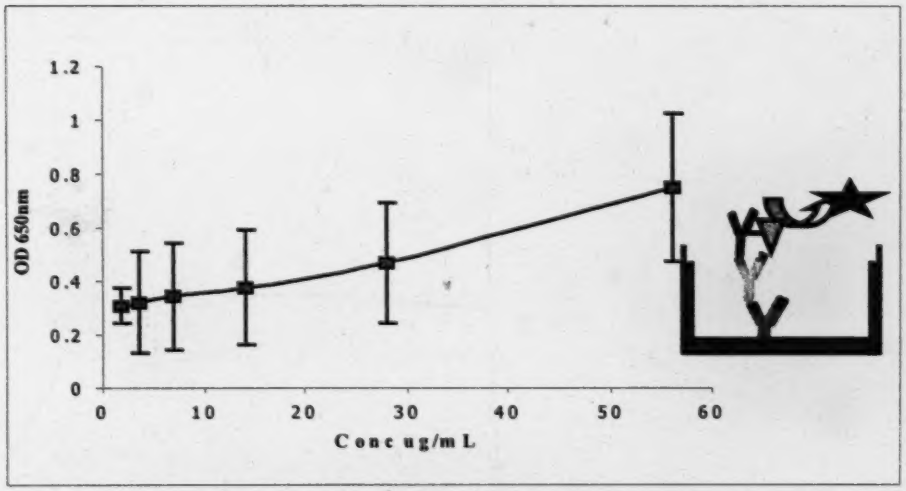


Figure 3.5 b: Goat anti-mouse on solid phase and goat anti-mouse IgG peroxidase as the reporter enzyme at 1:10,000

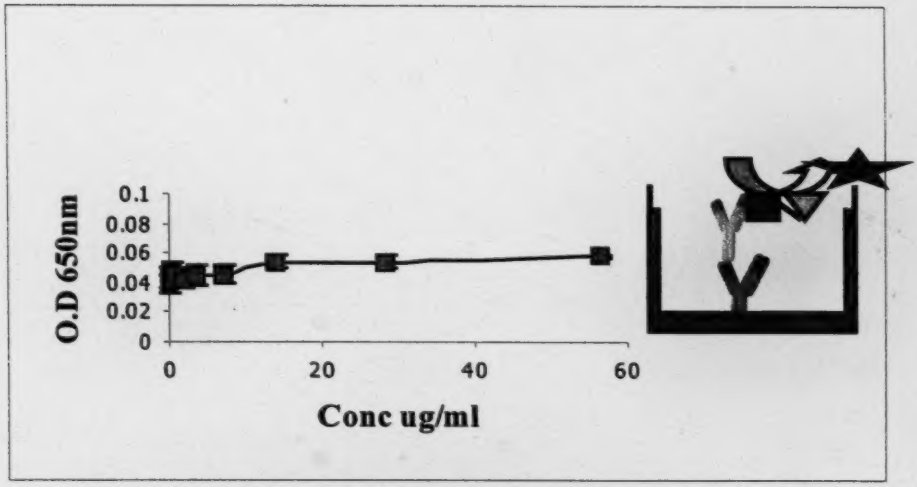


Figure 3.6 a: Goat anti-mouse IgG on the solid phase and protein-L peroxidase as the reporter enzyme at 1:2000

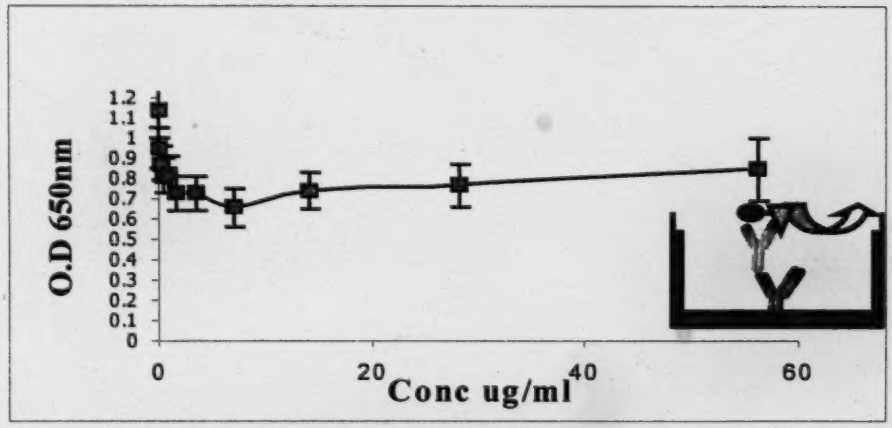


Figure 3.6 b: Goat anti-mouse IgG on the solid phase and protein-G peroxidase as the reporter enzyme at 1:2000

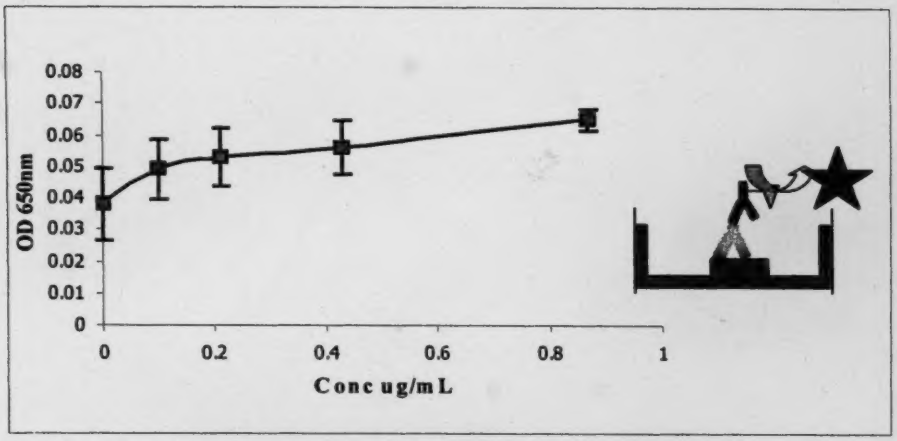


Figure 3.7 a: Protein-L on the solid phase and goat anti-mouse IgG peroxidase as the reporter enzyme at 1:10,000

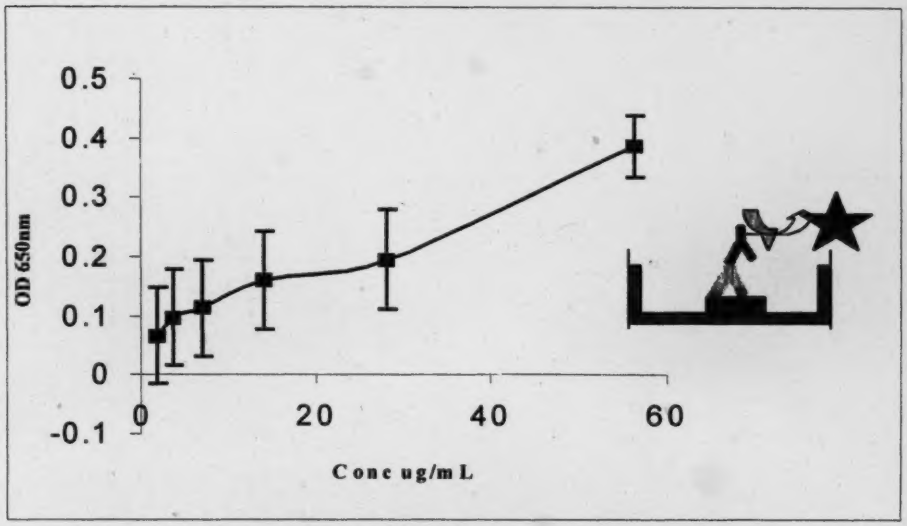


Figure 3.7 b: Protein-L on the solid phase and goat anti-mouse IgG peroxidase as the reporter enzyme at 1:10,000

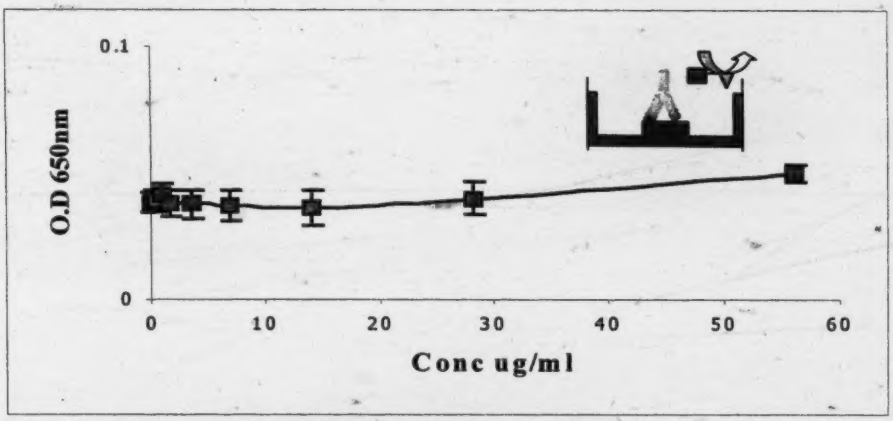


Figure3.8 a: Protein-L on the solid phase and protein-L peroxidase as the reporter enzyme at 1:2000

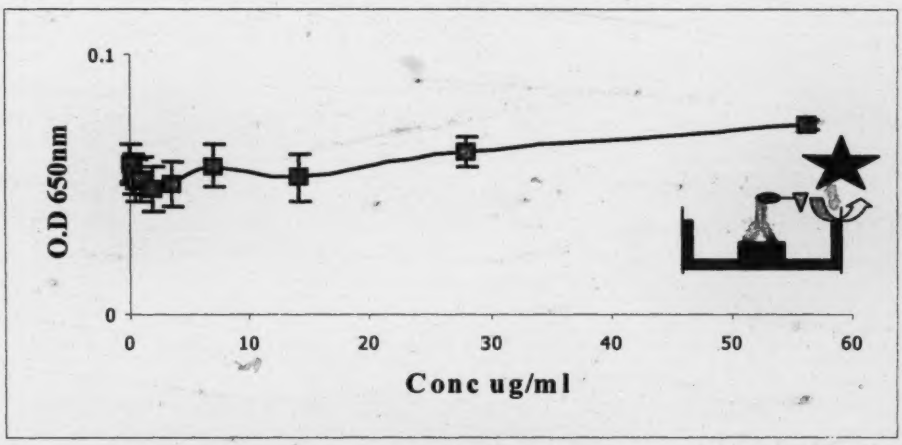


Figure3.8 b: Protein-L on the solid phase and protein-G peroxidase as the reporter enzyme at 1:2000

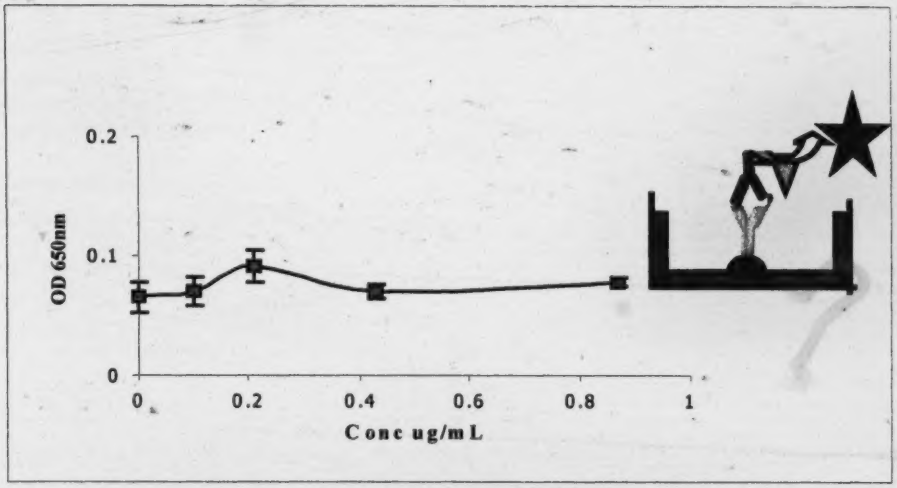


Figure 3.9 a: Protein-G as the solid phase and the goat anti-mouse IgG peroxidase as the reporter enzyme at 1:10,000

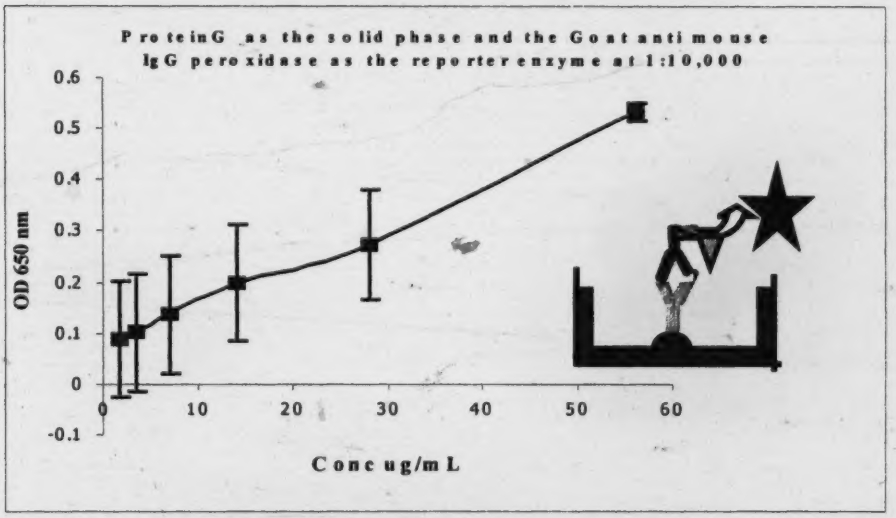


Figure 3.9 b: Protein-G as the solid phase and the goat anti-mouse IgG peroxidase as the reporter enzyme at 1:10,000

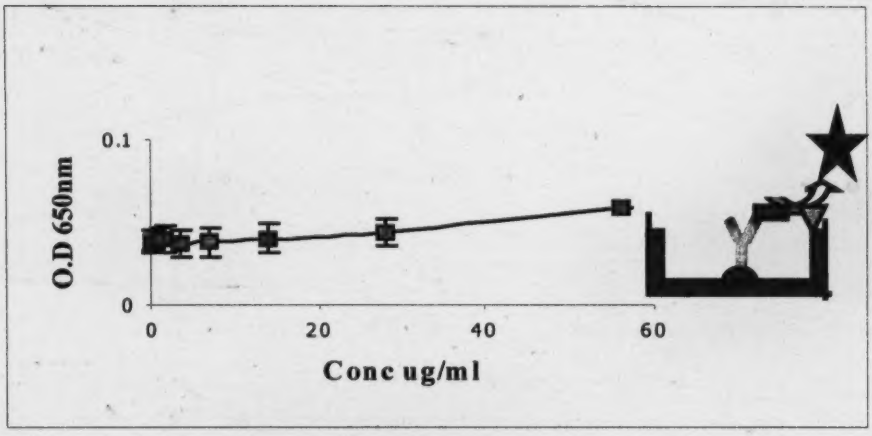


Figure 3.10 a: Protein-G as the solid phase and protein-L peroxidase as the reporter enzyme at 1:2000

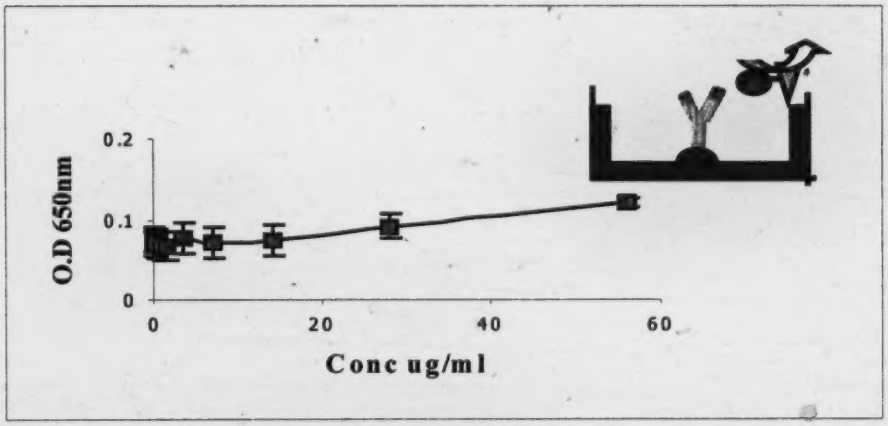


Figure 3.10 b: Protein-G as the solid phase and protein-G peroxidase as the reporter enzyme at 1:2000

In Protein-L solid phase with Protein-L peroxidase as the reporter enzyme (Figure 3.8 a), the values merely represented background signal. The solid phase Protein-L binds the variable regions of IgG and hence there is no free variable region of IgG to be bound by the protein-L peroxidase.

Similarly, in Protein-G solid phase with Protein-G peroxidase format, it also shows the signal values that are essentially background (Figure 3.10 b). Here the solid phase Protein-G binds the Fc of the IgG and the reporter enzyme has no chance to bind to the Fc of the IgG since it is already occupied by the solid phase Protein-G.

In goat anti mouse IgG Fc solid phase and protein-G peroxidase format, the results are background because most of the goat anti-mouse IgG binds the Fc, as well as other constant regions of the IgG and hence there is no chance for the protein-G peroxidase to bind the Fc of the IgG (Figure 3.4 a).

In Protein-G as the solid phase and Protein-L peroxidase format, the plot shows a plateau (Figure 3.10 a). This can be potentially due to low protein-L peroxidase enzyme titre and/or the cross reactivity between the bovine IgG in the BSA used as the blocking and subsequent conjugate enzyme diluent.

In the protein-L solid phase and protein-G peroxidase format, the curve took the shape of a plateau (Figure 3.8 b). Once again the possible reason could be that due to the cross reactivity between the bovine IgG in the BSA used as the blocking and subsequent conjugate enzyme diluent with the protein-G peroxidase.

In the goat anti-mouse IgG solid phase and protein-L peroxidase format (Figure 3.6 a), the curve has a plateau and this appeared to be due to the low activity of the commercial protein-L peroxidase enzyme conjugate.

In Goat anti-mouse IgG Fc solid phase and the protein-G peroxidase as the reporter shows a plateau curve as expected, because the IgG Fc was already occupied by the goat anti-mouse Fc on the solid phase (Figure 3.4a).

In general, the formats that were not successful were due to the 2% bovine IgG interference in the BSA used as the blocking agent and also the low enzyme activity of the protein-L and protein-G peroxidases. These eight formats were repeated with higher amounts of the protein-L and protein-G peroxidases and the ovalbumin as the blocking agent. Repetition of the 5 of 8 formats with the 1% ovalbumin as the blocking and enzyme conjugate diluent with higher amounts of the conjugate did not result in linear dose response (Figure 3.20-3.24). This has shown that the protein-L and protein-G conjugates were not functional in signal generation.

All the four formats that were successful (Figure 3.2), were repeated in the nano gram level concentrations, starting from 1.25 to 100 ng/mL to check the lowest detectable limit of these ELISA formats.

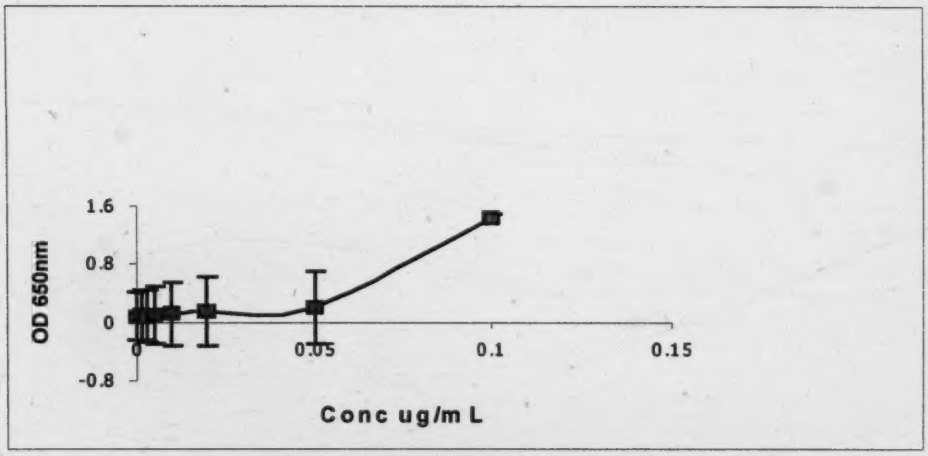


Figure 3.11: Goat anti-mouse IgG Fc as solid phase and goat anti-mouse IgG HRPO as the reporter enzyme 1:10,000

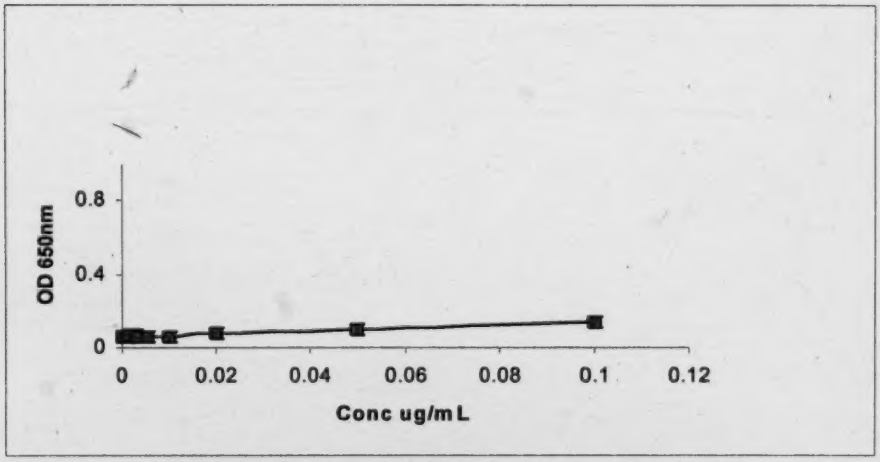


Figure 3.12: Goat anti-mouse as solid phase and goat anti-mouse IgG HRPO as the reporter enzyme 1:10,000

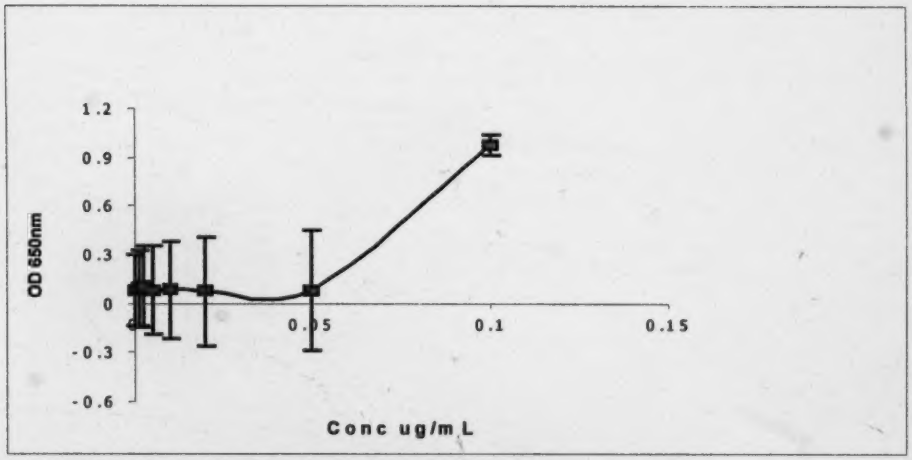


Figure 3.13: Protein-G solid phase and goat anti-mouse IgG HRPO as reporter enzyme 1:10,000

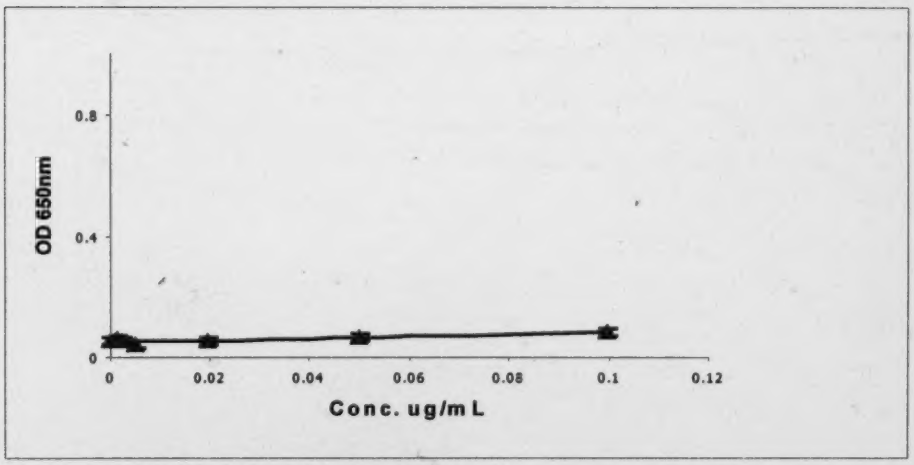


Figure 3.14: Protein-L solid phase and goat anti-mouse IgG HRPO as reporter enzyme 1:10,000

From these four plots (Figure3.11-3.14), at the nanogram range of the analyte, the two formats namely the Protein-L (Figure 3.14) and goat anti mouse IgG (Figure 3.12) as the solid phases exhibited no response at the nanogram levels, even though they have good signal response and linearity in the higher range of analyte concentrations (Figures 3.5 and b and 3.7 a and b). The two final formats, goat anti-mouse IgG Fc (Figure 3.11) and the protein-G (Figure 3.13) as the solid phase with goat anti-mouseIgG peroxidase as the reporter enzyme have showed good signal response linearity above 50 nanogram/mL. Among the 12 formats, only two formats remain as viable options as shown in fig 3.15.

	Antibody or Protein conjugated solution phase tracers		
	Protein-G peroxidase	**Protein-L** Peroxidase	**Goat α mouse IgG** peroxidase
Solid Phase Capture reagent			
Goat α mouse IgG	Goat α mouse IgG + Protein-G peroxidase	Goat α mouse IgG + Protein-L peroxidase	Goat α mouse IgG + Goat α mouse IgG peroxidase
Goat α mouse IgG Fc	Goat α mouse IgG Fc + Protein-G peroxidase	Goat α mouse IgG Fc + Protein-L peroxidase	Goat α mouse IgG Fc + Goat α mouse IgG peroxidase
Protein-G	Protein-G + Protein-G peroxidase	Protein-G + Protein-L peroxidase	Protein-G + Goat α mouse IgG peroxidase
Protein-L	Protein-L + Protein-G peroxidase	Protein-L + Protein-L peroxidase	Protein-L + Goat α mouse IgG peroxidase

Figure 3.15 Format of various sandwich assays for estimation of mouse IgG: The unsuccessful formats are shaded leaving further investigation of 2 formats in the last column.

3.3.2 Comparison of Bovine IgG and Mouse IgG responses at nano gram levels

The occurrence of 2% bovine IgG in the blocking and diluting agent (1% BSA), could be a potential cross-reacting and interfering agent, particularly when the ELISA is performed in the nanogram levels of analyte estimation. In order to verify the cross reactivity, we chromatographed the bovine serum on the protein-G column and obtained relatively pure bovine IgG. With bovine IgG and mouse IgG as the standards, the formats with protein-G as the solid phase with goat anti-mouse IgG-HRPO as the reporter have been carried out with 1% ovalbumin as the blocking and diluting agents.

The two formats (Figure 3.16 and 3.17) shows that ELISA responses with mouse IgG and the bovine IgG are similar. This confirms the cross reactivity of protein-G with bovine IgG and demonstrates this component as interference in the very low range of analytes. This observation is not surprising as the protein-G has the same affinity towards the bovine IgG and mouse IgG (Akerstrom,B et al., 1986). So in conclusion, this format cannot be used in the nanogram levels of ELISA with the BSA as the blocking and diluting agent, because at the nanogram levels, the nanogram concentrations of the analyte (mouseIgG) has to compete with the microgram concentration of the bovine IgG. Alternatively, the blocking and diluting reagents could be processed on a protein-G column to remove the interfering factors.

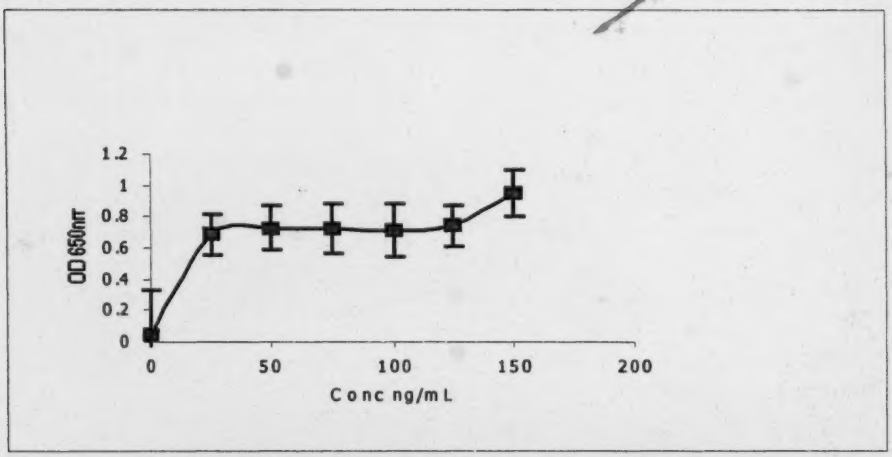


Figure 3.16: Mouse IgG at nano gram level with protein-G as the solid phase and goat anti-mouse IgG peroxidase as the reporter enzyme at 1:10,000

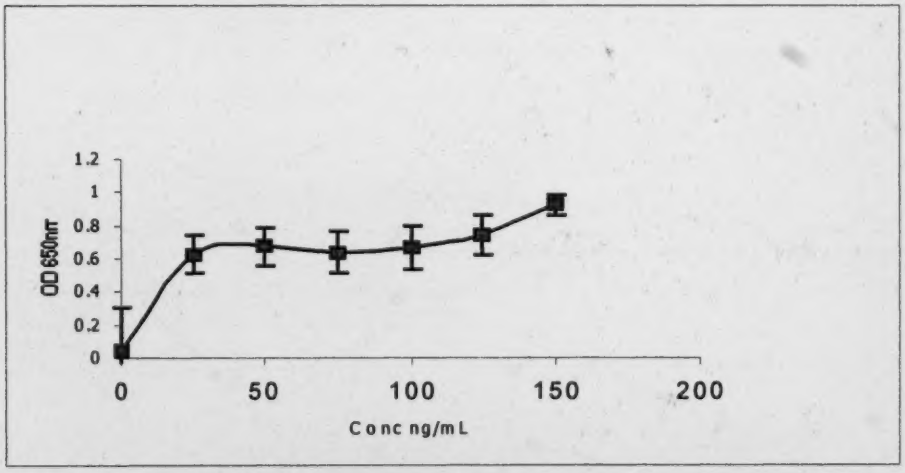


Figure 3.17: Bovine IgG at nano gram level with protein-G as the solid phase and goat anti-mouse IgG peroxidase as the reporter enzyme at 1:10,000

The ELISA plots (Figure 3.18 and 3.19) show that there is no signal generation with the bovine IgG, while the mouse IgG estimation in this format at the nanogram concentrations exhibits a linear dose relationship. This also demonstrates that there is no cross reactivity between goat anti mouse Fc and the bovine IgG. Hence this format is the best assay for the quantitative estimation of mouse IgG in comparison to other formats studied. This format can be used as a universal ELISA tool for the quantification of the murine IgG hybridomas in the biological sample of interest. This format has shown a good linear increase in the O.D from 0 to 3.51 µg/mL concentration. At the higher concentrations of mouse IgG the signal plateaued due to the saturation of the reaction between mouse IgG and the solid phase as well as the solution phase tracer. Hence the mouse IgG concentration which has to be quantified by this ELISA format should be performed with the appropriate dilution to be within the range of the standard curve.

Among the various ELISA format choices, finally it was able to logically derive the best combination, with the format of goat anti-mouse IgG Fc solid phase and the goat anti-mouse IgG peroxidase as the most reliable with no cross reactivity to bovine IgG.

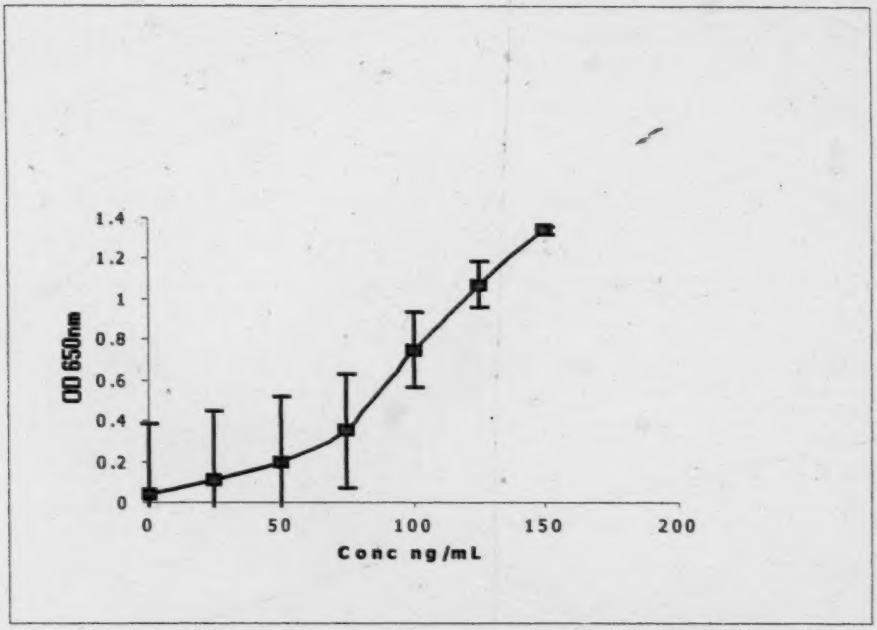


Figure 3.18: Mouse IgG at nanogram level with goat anti-mouse Fc as the solid phase and goat anti-mouse IgG peroxidase as the reporter enzyme at 1:10,000

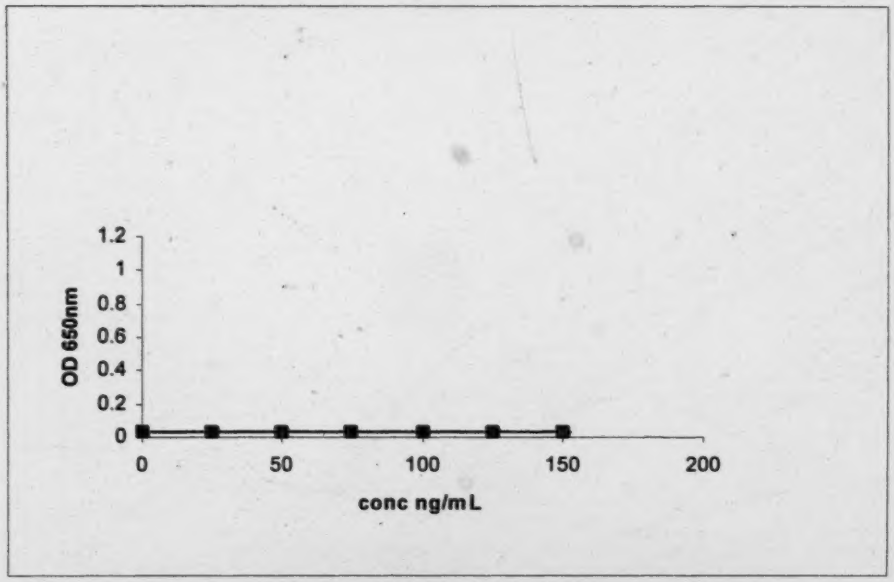


Figure 3.19: Bovine IgG at nanogram level with goat anti-mouse Fc as the solid phase and goat anti-mouse IgG peroxidase as the reporter enzyme at 1:10,000

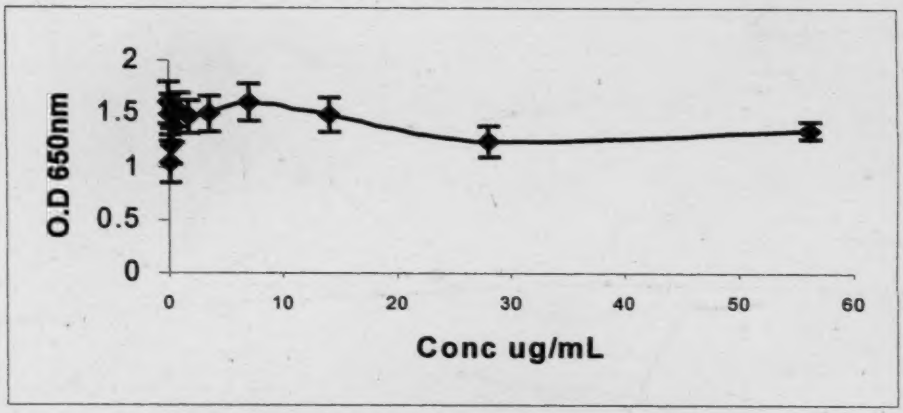


Figure 3.20: Goat anti-mouse IgG as the solid phase and protein-G peroxidase as the reporter enzyme 1: 1000

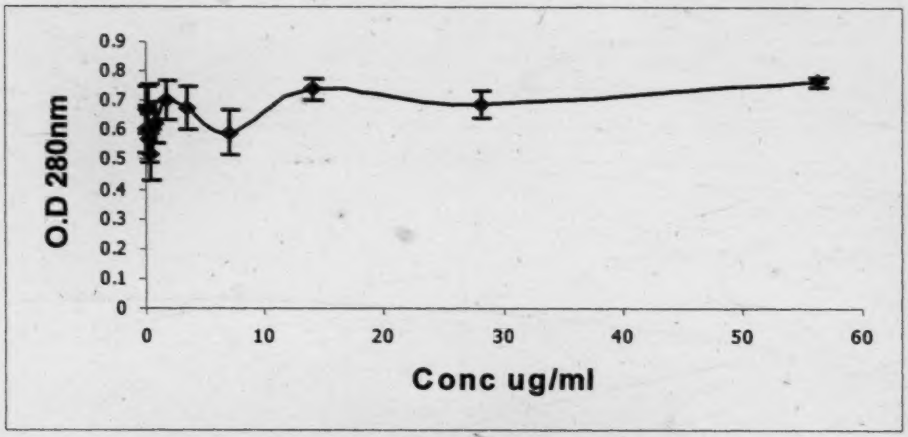


Figure 3.21: Goat anti-mouse IgG as the solid phase and protein-L peroxidase as the reporter enzyme 1: 1000

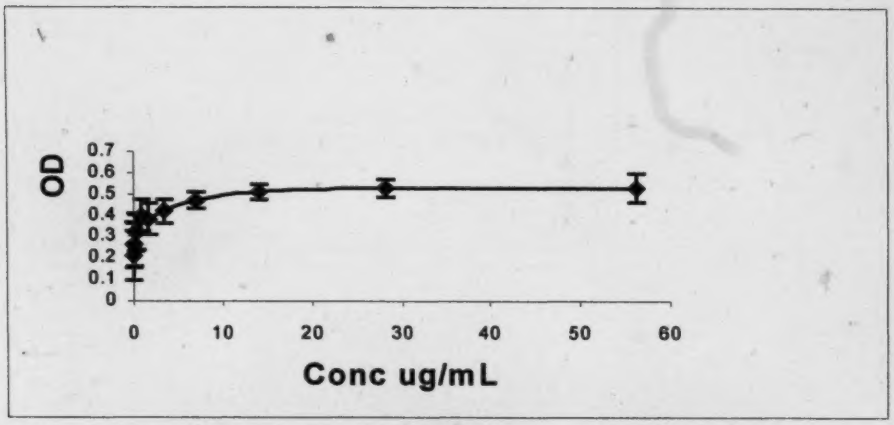


Figure 3.22: Goat anti-mouse IgG Fc specific as the solid phase and protein-L peroxidase as the reporter enzyme at 1:1000

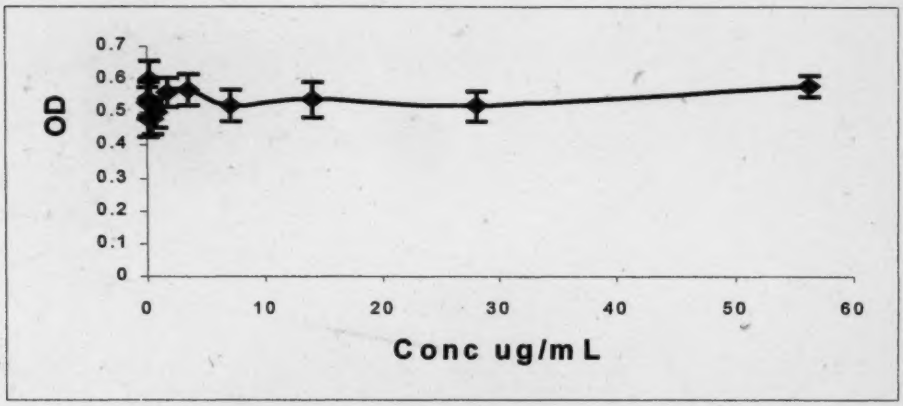


Figure 3.23: Protein-G as the solid phase and protein-L peroxidase as the reporter enzyme at 1:1000

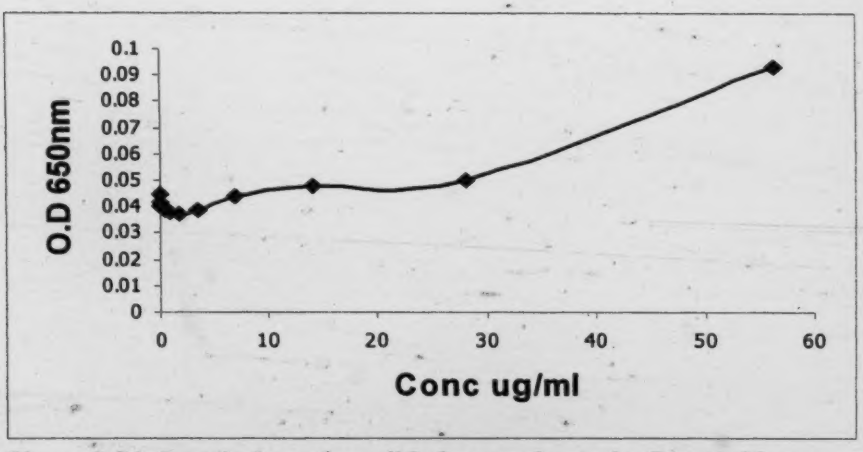


Figure 3.24: Protein-L on the solid phase and protein-G peroxidase as the reporter enzyme at 1:1000

3.3.3 Evaluation of the bioreactor Mab harvests

The selected most optimal format, goat anti-mouse IgG Fc solid phase and goat anti-mouse IgG peroxidase was explored for its applicability to quantitate the Mab concentrations in the bioreactor raw culture supernatants extensively described in chapter 2. In this study, a standard curve was performed simultaneously with the raw culture supernatants as analyte in the same ELISA plate. The raw culture supernatants were diluted 10 times and the dilution factor was latter multiplied with the concentrations obtained from the standard curve for each bioreactor sample. The line equation and the linear regression of the standard curve are shown in the figure 3.25.

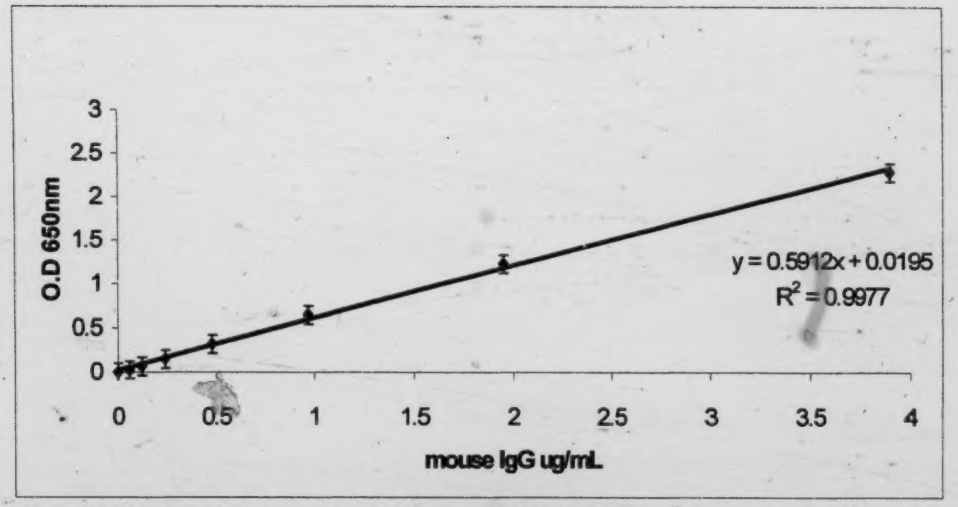


Figure 3.25: Evaluation of Mab harvests with the goat anti-mouse IgGFc solid phase and goat anti-mouse IgG peroxidase as the reporter 1:10,000.

The table 3.1 shows the yields calculated from the ELISA standard curve and the yields obtained from the Protein-G purification of the Mab harvests.

Table 3.1: Comparison between the yields calculated from ELISA and UV_{280nm}

Bioreactor Harvests	Yields calculated from ELISA curve	Yields obtained from the Protein-G purification
I-Mab-H2 RPMI	6.9±0.0075	7.6
I-Mab-H2 SFM	5.9±0.0032	6.2
MP H0.5 RPMI	14.0±0.0037	14.2
I-Mab H2 DMEM	14.5±0.0047	14.8
I-Mab H2 PFC	22.7±0.0050	22.8
RPMI-culture flask	4.0±0.0047	4.7
I-Mab H2 B.D	16.34±0.0079	16.6

From the table above, it is noticed that the yields calculated from the ELISA curve were always lower than that of the yields obtained from the Protein-G purification. This is

expected since, there would be a concomitant co-purification of the bovine IgG (in culture media) along with the murine Mab during the Protein-G purification. Trebak M et al., 1999 have reported that there was a 0.6% of bovine IgG contamination in serum supplemented cultures.

3.3.4 Spike and recovery of the mouse IgG in Human Serum

As described in chapter 1, Mabs have been used *in-vivo* for therapeutic applications, I investigated the potential utility of the mouse IgG ELISA to study the *in-vivo* pharmacokinetics of mouse monoclonals in a simulated therapeutic setting. This spike and recovery experiment, to look into the feasibility of measuring the mouse antibody concentrations in the human blood was not encouraging since the recovery was not quantitative. It can be visualized from the comparative plots in figure3.26, that there is a decrease in signal, when the mouse IgG was spiked and estimated in human serum. This could possibly be the serum matrix effect. Perlmann, H. and Perlmann, P.(1994) have reported that, when estimating substances in serum or plasma, serum contents generally suppress the antigen-antibody reaction this is called as the matrix phenomenon or effect. In addition, serum contains significant human component potentially cross-reacting with the ELISA format. Further optimization needs to be performed if the desire is to establish an assay specific for mouse IgG in human serum.

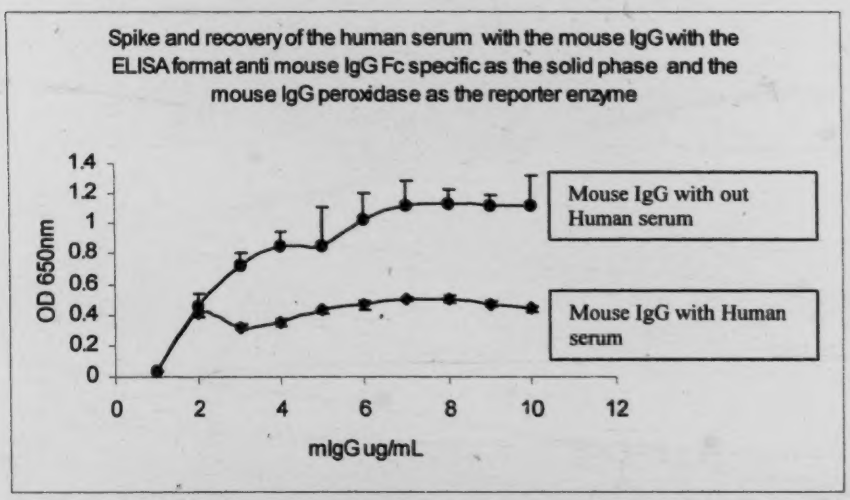


Figure 3.26: Spike and recovery of the human serum with the mouse IgG,using the ELISA format goat anti-mouse IgG Fc specific as solid phase and goat anti-mouse IgG HRPO as the reporter 1:10,000.

3.4 Conclusion

The development of sensitive SPIAs depend not only on the format design, but it also depends on various factors such as the influence of the reagents such as the crossreactivity of the blocking, diluting buffers, the titre of the conjugate enzyme and also the effect of the interfering agents in the analyte sample. The analyte sample may have some extraneous substances, which could also suppress the antibody interaction or allevate the signal generation. In an attempt to develop a quantitative ELISA, it was very important to study the effect of the potential interfering substances in the assay. In our study, the screening of the 12 different formats could have been reduced, if the effect of the bovine IgG on few of the formats was known. The outcome of this extensive study shows that bovine IgG can be a key interference. This led me to develop an assay that is highly specific to only mouse IgG for evaluation of bioreactor supernatants. My recommendation is for the of the use of Goat anti-mouse IgG Fc solid phase and Goat anti-mouse IgG peroxidase as tracer with 1% BSA as the blocking and conjugate enzyme diluent for a generic mouse IgG ELISA. This assay can be utilized for the estimation of the murine Mab secretion levels in biotechnology applications. The use of this assay in the estimation of murine antibody in human serum was demonstrated and further optimization need to be explored in the future.

CHAPTER-IV
Downstream Processing of Monoclonal antibodies From Culture Supernatant

4.1 Introduction

Purifying IgG or Mabs from serum or ascities can be performed by a number of methods including ethanol precipitation, salt precipitation, PEG precipitation, caprylic acid precipitation, ion exchange chromatography or affinity chromatography (Current Protocols in Immunology). Among these methods, only affinity chromatography gives high purity of IgG in one step.

The technique of affinity relies upon the ability of proteins to recognize and bind to other molecules (ligands) in a specific and reversible manner (C.R Merril, 1990). Affinity chromatography uses an adsorbent comprising a porous matrix to which the ligand is attached. The attachment or bonding is performed so that the immobilized ligand is still able to interact with the protein. An affinity separation is then performed by passing the impure protein over the adsorbent, whereby at which juncture, the target protein is adsorbed, while allowing contaminants to pass through without hindrance. Following adsorption, the adsorbent is washed to remove residual contaminants and the bound protein eluted in a pure form. Elution is normally achieved by changing the buffer or salt composition so the protein can no longer interact with the immobilized ligand. Elution methods may be of two groups; selective and non-selective. Non-selective elution methods rely on physico-chemical changes such as pH, ionic strength, solvent polarity etc. Selective elution methods use more specific ways of desorbing the bound protein.

Addition of a competing ligand or its analogues or removal of a complexing agent are often used in selective elution strategies.

Mabs differ in their biochemical and their ligand characteristics, making it necessary to develop individual protocols for their purification. However, due to the existence of common constant domains (Fc and those of the light chains), group specific affinity chromatography can be utilized for Mab purification (Akerstrom.B 1989).

In this work, three different types of affinity chromatography techniques were used to purify the Mabs such as Protein-G affinity chromatography, Biotin-agarose affinity chromatography (Cao et al., 1998) and Dye-ligand (mimetic blue ligand) affinity chromatography (Xu et al., 1997). The Mab harvests were purified on the appropriate chromatography columns, as per their specificities.

4.2 Materials and Methods

4.2.1 Materials

Protein-G affinity column: A lyophilized powder of protein-G immobilized on 4%-beaded agarose was obtained from Sigma St.Louis U.S.A.

Biotin affinity column: A 2mL Biotin immobilized on 4% agarose was obtained from Sigma St.Louis U.S.A.

Mimetic blue affinity column: A 25mL of the mimetic blue affinity matrix (Mimetic Blue AP A6XL) was obtained from the Prometic Biosciences, Canada.

Culture Supernatants: Bioreactor culture supernatants were collected from the following cell lines: A4A1 (anti-VEE): Secretes mouse IgG monoclonal antibody against Venezuela equine encephalitis. Obtained from DRES-DND (Suffield). 11D2(anti-WEE): Secretes mouse IgG monoclonal antibody against Western equine encephalitis. Obtained from DRES-DND (Suffield). 5B4D6(anti-VEE): Secretes mouse IgG monoclonal antibody against Venezuela equine encephalitis. Obtained from DRES-DND (Suffield). P54 (anti-Biotin): Secretes mouse IgG monoclonal antibody against Biotin. P106 (anti-M13 x anti-AP): Secretes bispecific monoclonal antibody, with one arm specific against M13 and the other arm specific against Alkaline Phosphatase. H.B 191(anti-mouse NK cell): Secretes IgG monoclonal antibody against mouse Natural killer cells, obtained from Dr.Terry Allen lab, Pharmacology, University of Alberta. FMC-63 (anti-CD-19): Secretes IgG monoclonal antibody against CD-19, obtained from Dr.Terry Allen lab, Pharmacology, University of Alberta.

Purification Accessories: 10mL syringe Becton Dickinson, 18.5 gauge needle from Becton Dickinson, Polyethylene frit from Bio Rad, double silencing stopper from Fisher, Disposible serological fraction collection tubes from Falcon, vacuum grease from Masterflex , peristaltic pump from Pharmacia , Masterflex tubing from Masterflex, dialysis tubing from Spectrum labs and Polyethylene glycol , Ammonium sulphate were obtained from BDH. Milli Q –deionised 18-m ohm water was used in most experiments. UV-spectrophotometer from Becton Dickinson, USA was used for A_{280nm} absorption measurements.

Buffers and solutions: PBS pH 7.0; 0.1M glycine-HCl pH 2.8; 0.1M Tris-HCl pH 9.0; 0.01M Tricine Buffer pH 8.5; 0.01M Tricine Buffer with 0.01M Potassium phosphate; Sodium azide 0.05%; 0.1N HCl; 1M NaOH; 0.3M d-Biotin pH 2.8; alkaline phosphatase solution in Tricine Buffer pH 8.5. Chemicals used for making the buffers such as, sodium hydroxide, sodium di-hydrogen phosphate (anhydrous), di-sodium hydrogen phosphate (anhydrous), sodium chloride, potassium phosphate were obtained from BDH U.S.A. Glycine, tris, tricine , and sodium azide were obtained from Life technologies, Ontario Canada. d-biotin and alkaline phosphatase were obtained from Sigma Scientific St.Louis U.S.A .

SDS PAGE: The chemicals used for SDS PAGE were ACS certified reagents obtained as follows:

40% acrylamide, TEMED, protein molecular weight marker, coomassie brilliant blue crystals were obtained from Bio Rad USA. Ammonium per sulphate, butanol, glycerol, glacial acetic acid were obtained from BDH U.S.A. Bromophenol blue, whatmann papers were obtained from Fisher scientific.

4.2.2 Methods

Details of the column preparations are presented below

4.2.2.1 Protein-G affinity chromatography

IgG can be purified by ammonium sulfate precipitation followed by size-exclusion chromatography. This is the least expensive option available for purification of antibodies. Protein-A and Protein-G affinity chromatography are currently the most common methods for purifying antibodies. Protein-G affinity chromatography can be carried out using a commercially produced kit from Sigma USA, using standard binding and elution protocols. Protein-G (Akerstrom and Bjorck, 1986) has a binding profile opposite to that of protein-A with respect to the pH. Not all IgG molecules bind to protein-A. Many mouse IgG subclass antibodies will not bind to Protein-A.

Protein-G is a cell wall protein (originally obtained from Type G Streptococci) that binds the Fc region of immunoglobulinG (IgG) with high affinity. This product is prepared from the recombinant Protein-G, which is a truncated protein that retains only the Fc binding sites. It does not bind to the Fab, albumin and the cell membranes. The Binding capacity of the column is 25mg/mL of the human IgG.

Since the cell lines A4A1, 11D2, 5B4D6, HB 191 and FMC-63 are all murine IgG Mabs, Protein-G was a convenient matrix to purify these Mabs by the affinity chromatography . Reliability of the column was also tested by purifying the mouse IgG periodically.

Column preparation

The protein-G lyophilized powder was hydrated by placing the resin in deionized water , approximately 25mL per gram of resin for 30 minutes. The gel (2mL) was then loaded into a 10mL BD syringe fitted with a frit at the bottom. The resin bead was repeatedly

washed with deionised water (about 50mL) to remove the bacteriostats and the stabilizers from the resin. The column bed was latter equilibrated with PBS buffer pH 7.0. The column was usually preserved in PBS containing 0.05% Sodium azide and stored at 0-5 °C.

Protein-G affinity purification methodology

Equilibration: The protein-G affinity column was washed with about 25 mL of PBS, pH 7.0. This buffer is called as the starting buffer or loading buffer or the equilibration buffer. This equilibration step was performed in order to remove the bacteriostat-sodium azide from the column.

Dilution: The culture fluid was clarified to be particulate free by a centrifugation step.The pH of the cell culture supernatant was the same as that of the equilibration buffer and the supernatant was also diluted (1:2 dilution) with the same equilibration buffer.

*Loading:*The diluted culture supernatant was then loaded onto the column in cold room with the aid of the peristaltic pump. The flow rate was set at 0.5mL per minute to facilitate maximal binding and recovery.The unbound was collected in a reservoir.

*Washing:*The column was washed with PBS pH 7.0. The UV spectrophotmeter was set to zero optical density at 280nm by using PBS as the blank. The optical density of the wash fraction was read at 280nm to ensure that all other extraneous proteins were eluted and UV absorbance returned to baseline.

Elution: The UV spectrophotometer was adjusted to read blank with a mixture of 550 µL of 0.1M Glycine-HCl and 450µL of 0.1M Tris-HCl pH 9.0.The bound IgG on the column was eluted with 0.1M Glycine-HCl pH 2.8. This low pH would dislodge the antibody from the bound ligand and pass through the column as eluates along with the eluent. To

avoid denaturation and to preserve the activity of acid labile IgG, 450 μL of 0.1M Tris-HCl pH 9.0 was added to those tubes destined to collect fractions containing Mabs. This ensures that, the final pH of the sample was approximately neutral. The column was re-equilibrated to the neutral pH and then stored by adding 0.05% sodium azide to the slurry. A schematic representation of the purification steps are shown in the figure 4.1.

Resin regeneration

The resin was cleaned to remove non-specific proteins periodically by washing the slurry in 0.1N HCl and subsequently equilibriated and stored with PBS containing 0.05% sodium azide.

4.2.2.2 Biotin-agarose affinity Chromatography

Biotin is a coenzyme also called as vitamin-H, it has high affinity towards the avidin. The Mab produced by the cell line P54 also bind biotin albeit at lower affinity (Cao et al.,1998) and has an anti biotin property. So a Biotin-agarose affinity column was used as a tool for the purification of the antibody against biotin. Resin preparation, resin regeneration and the protocol of the purification methodology is exactly similar to that of the protein-G affinity chromatography, but the only difference lies in the elution buffer. The column (2mL in size) was competitively eluted with the 0.3-mM d-biotin in glycine-HCl, pH 2.8. This elution procedure is a selective technique, where the elution buffer is a competitive ligand in excess that dislodges the antibody bound to the column. A schematic representation of the purification is shown in figure 4.2.

4.2.2.3 Mimetic blue dye-ligand affinity chromatography

Mimetic blue AP A6XL is a synthetic affinity adsorbent developed specifically by Prometic Biosciences for the purification of alkaline phosphatase and alkaline phosphatase immunoconjugates. The Mimetic blue AP ligand consists of a blue chromophore linked to a phosphate analogue. This ligand displays a high affinity for alkaline phosphatase and alkaline phosphatase immunoconjugates. The affinity ligand is bonded onto a 6% cross-linked agarose by a highly stable bonding method, which is resistant to treatment with 1M NaOH. The use of NaOH cleaning procedures ensures that Mimetic Blue AP A6XL can be used repeatedly for purification of relatively crude protein extracts. The binding capacity of the column is typically 2mg/mL. The cell line P106 is a bispecific quadroma Mab that has specificity against M13 bacteriophage and alkaline phosphatase. Since one arm of this Mab can bind to alkaline phosphatase, this affinity medium was exploited for the purpose of purifying this Mab.

Resin Preparation

Mimetic ligand AP A6XL adsorbent was supplied as aqueous slurries in ethanol/0.1M NaCl (25:75(v/v)). This affinity medium slurry was degassed prior to column packing. The bed was washed with several volumes of equilibration buffer, 0.1M Tricine buffer pH 8.5. The bed volume of the gel was 25mL.

Mimetic Blue affinity Purification Methodology

Antibody Sample Preparation: The P106 is an anti-Alkaline phosphatase Mab, the raw culture supernatant was subjected to 50% ammonium sulfate precipitation and the precipitate was then dialyzed (100 volumes with 3 changes) extensively against 0.1M Tricine buffer pH 8.5 to remove the traces of phosphates from the antibody sample, since

it would interfere in affinity chromatography. The precipitate was then filtered and subjected to the mimetic blue affinity purification.

The purification methodology was performed as per the following steps.

- *Equilibration:* The mimetic blue affinity column was equilibrated with 0.01M Tricine buffer pH 8.5.This buffer is called as the starting buffer or loading buffer or the equilibration buffer. This equilibration step was performed in order to remove the bacteriostat-sodium azide from the column.
- *Alkaline phosphatase incubation:* To this equilibriated column 10mL of 5mg/mL of alkaline phosphatase in 0.01M Tricine buffer pH 8.5 was added and incubated overnight at 4°C to allow for maximal binding of alkaline phosphatase.
- *Washing:* The column was now washed with 0.01M Tricine buffer pH 8.5 to remove the unbound alkaline phosphatase.
- *Loading:* The antibody sample was subsequently loaded and incubated with the column at room temperature for 1hour.
- *Washing:* The UV spectrophotometer was set to blank at 280nm with 0.01M Tricine buffer pH 8.5. The column was washed with 0.01M Tricine buffer pH 8.5 to remove the unbound antibodies and other proteins. The unbound fractions included the M13 hybridoma antibodies. The washing was continued until the UV absorbance returned to the baseline.
- *Elution:* The UV spectrophotometer was set to blank with 0.01M Tricine buffer containing 0.01M potassium phosphate. The unbound Mab/AP immune complexes were eluted by 0.01M Tricine buffer containing 0.01M potassium phosphate (Linder 1989). The use of this mild elution by phosphate is an effective specific elution

method. The fractions eluted did not only contain the P106 bispecific antibody-AP, but also contained the hybridoma of the anti alkaline phosphatase-AP. The column was washed by the 0.01M Tricine buffer 8.5 and then about 2 ml of the same buffer with 0.1% sodium azide was added as a bactriostatic to the column slurry. The schematic of purification methodology is shown in figure 4.3.

- *Regeneration of the Column:* Washing the column with 0.1N NaOH regenerated the column as per the manufacturer's guidelines.

4.2.2.4 Post Purification Processing of the Mabs

The eluted fractions were pooled and then dialyzed against PBS pH 7.0 (in case of P106, the dialysis was against 0.01M Tricine buffer) with 3 changes at 0-5°C temperature with moderate stirring. The dialysed antibody solution was then concentrated by PEG. In the case of P54 antibody, the dialysis procedure also removes the biotin bound to the antibody. The OD of the Mab concentrate was read at 280 nm and based on the extinction coefficient calculation of OD_{280nm} 1.5 equals to 1mg/mL, using a 1 cm path length, (Pace et al., 1995) the antibody concentration was determined and stored at -20°C.

4.2.2.5 SDS-PAGE Procedure

Preparative solution or running gel: The preparative solution for SDS PAGE was made of the following composition in the following sequence 3.75 mL of 40% acrylamide; 3.75 mL of 4X Tris/SDS pH 8.8; 7.5mL of deionised milli-Q water; 50 µL of 10% ammonium persulphate; 10 µL of TEMED.

Stacking Solution or loading gel: Stacking solution was prepared by the following components in the following sequence- 0.5mL of 40% acrylamide; 1.25mL of 4X

Tris/SDS pH6.8; 3.75mL of milliQ water; 25 µL of 10% ammonium per sulphate; 5 µL of TEMED.

6X SDS/sample buffer: Prepared by the mixture of 7mL of 4X Tris-Cl/SDS, pH 6.8, 3.8g of glycerol (~3 mL), 1g ofSDS, 0.93g of Dithiothretiol (DTT), 1.2mg of bromophenol blue, add water upto 10mL and stored in 0.5mL aliquotes at -20°C.

5XSDS/electrophoresis buffer: Prepared by the mixture of 15.1 g Tris base, 72.0g of glycine, 5gm SDS, water upto 1000mL, diluted to 1X for working solution.

Coomassie brilliant blue solution: 0.25 gm of the coomassie blue crystals were dissolved in 90mL of methanol:water (1:1 v/v) and 10mL of glacial acetic acid, the solution was filtered through a Whatmann No 1 filter.

Destaining solution: Prepared by mixing 25mL methanol + 7mL acetic acid upto 100ml with distilled water.

The stacking plates were stacked leakproof on the Bio Rad SDS PAGE mounting apparatus to ensure it was also leak proof by filling with water. The running gel was loaded between the stacking plates, the preparative solution was set aside for 20 minutes to allow the solution to turn to gel. To prevent the gel from drying, water saturated butanol was added to prevent the drying of the gel. After the gel was formed, the butanol was removed and then stacking solution was added. Then immediately a 10 well comb was inserted into the stacking solution and allowed to set, later the comb was removed when the solution has solidified. The electrophoresis chamber was filled with the 1X SDS/electrophoretic buffer. Into each eppedrof, 40 µL of the antibody or culture supernatant samples were taken and 10 µL of the 6X SDS/sample buffer was added. In a separate eppendrof, 12 µL of the marker and 10 µL of the 6X SDS/sample buffer were

added. These were allowed to boil in the boiling water bath for 10 minutes to allow complete dissociation of the protein subunits. The boiled samples were loaded at the rate of 20 μL for each well and 6μL of marker was loaded into the marker well. The gel was then run under a current of 60 milliamps for 45 minutes. The gels were submerged in the Coomasie blue dye solution for 2 hours with shaking, in order for the protein bands to absorb the stain. Subsequently, the gel was put in a destaining liquid with shaking for 2 hours. The gel was then sealed in a plastic film and scanned for data acquisition.

Figure 4.1a

Schematics of Protein-G affinity purification technique

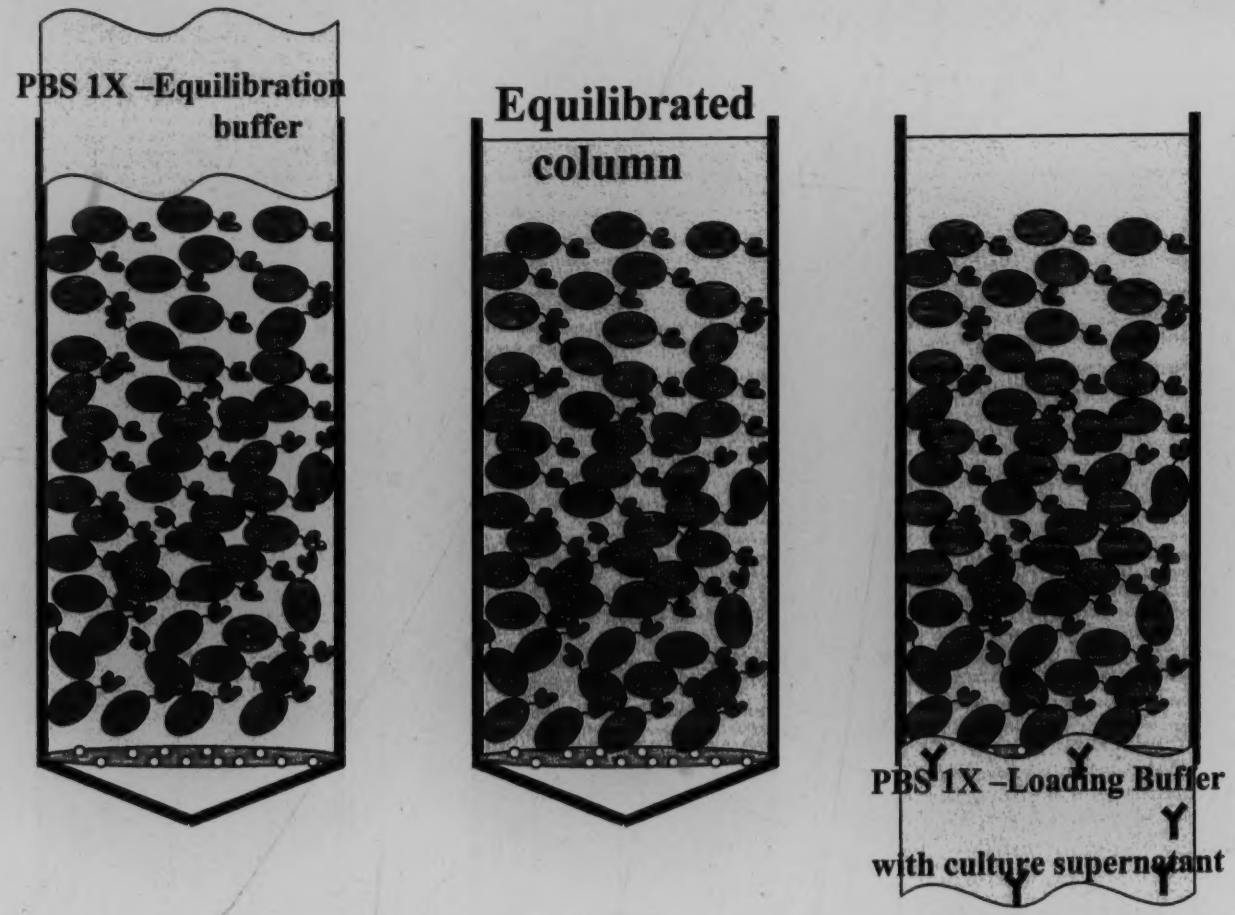


Figure 4.1 b

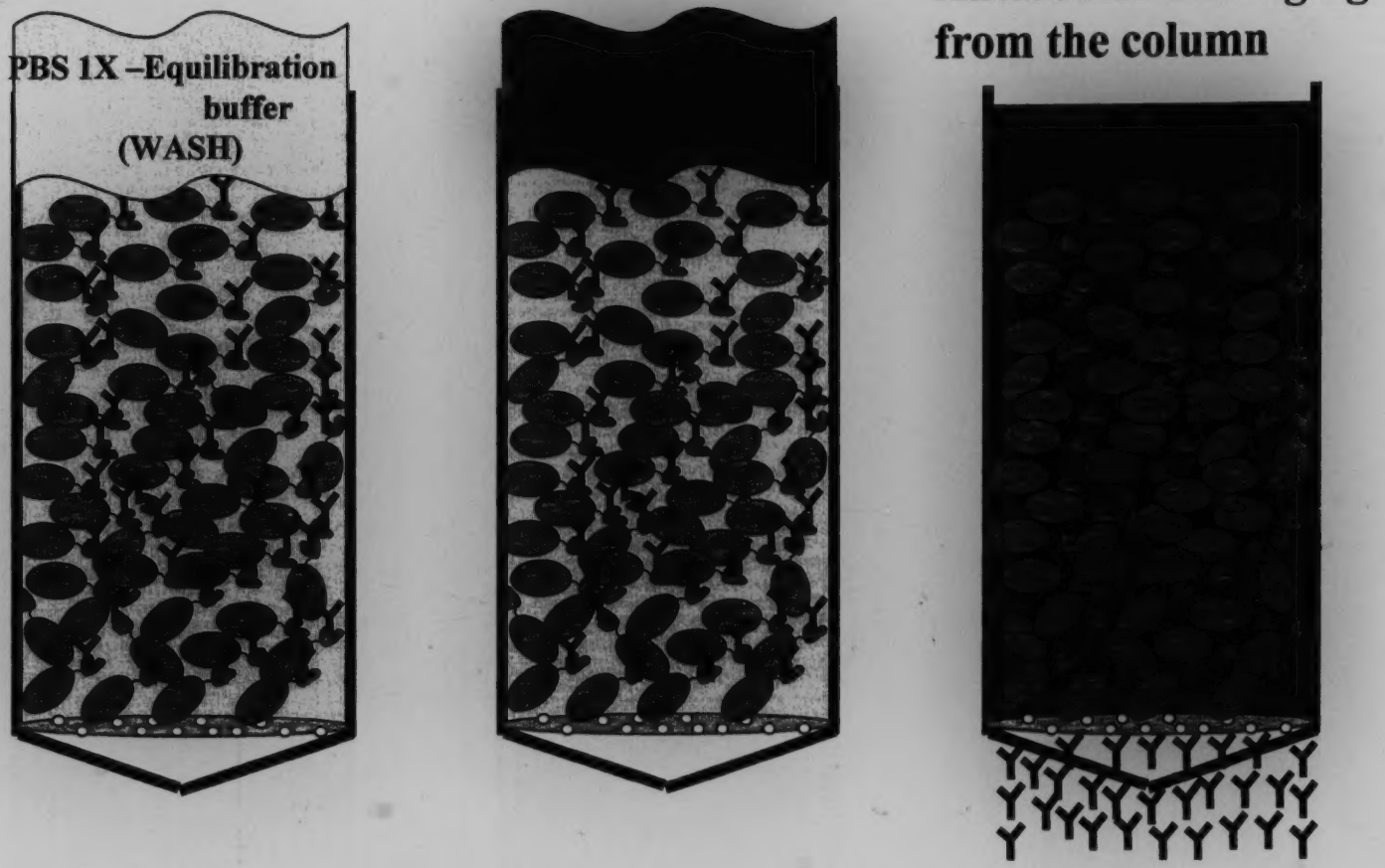

PBS 1X –Equilibration buffer (WASH)
Antibodies dislodging from the column

Figure4.2a

Schematics of Biotin-affinity purification

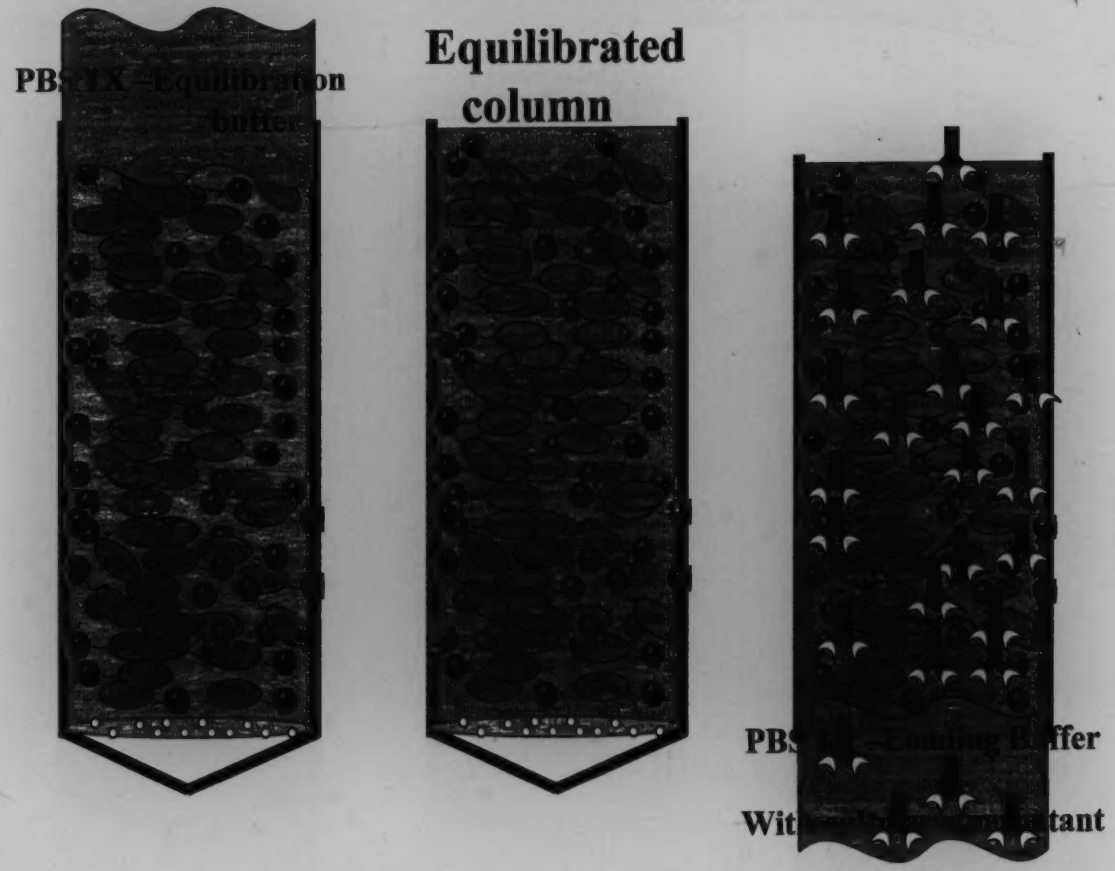


Figure 4.2 b

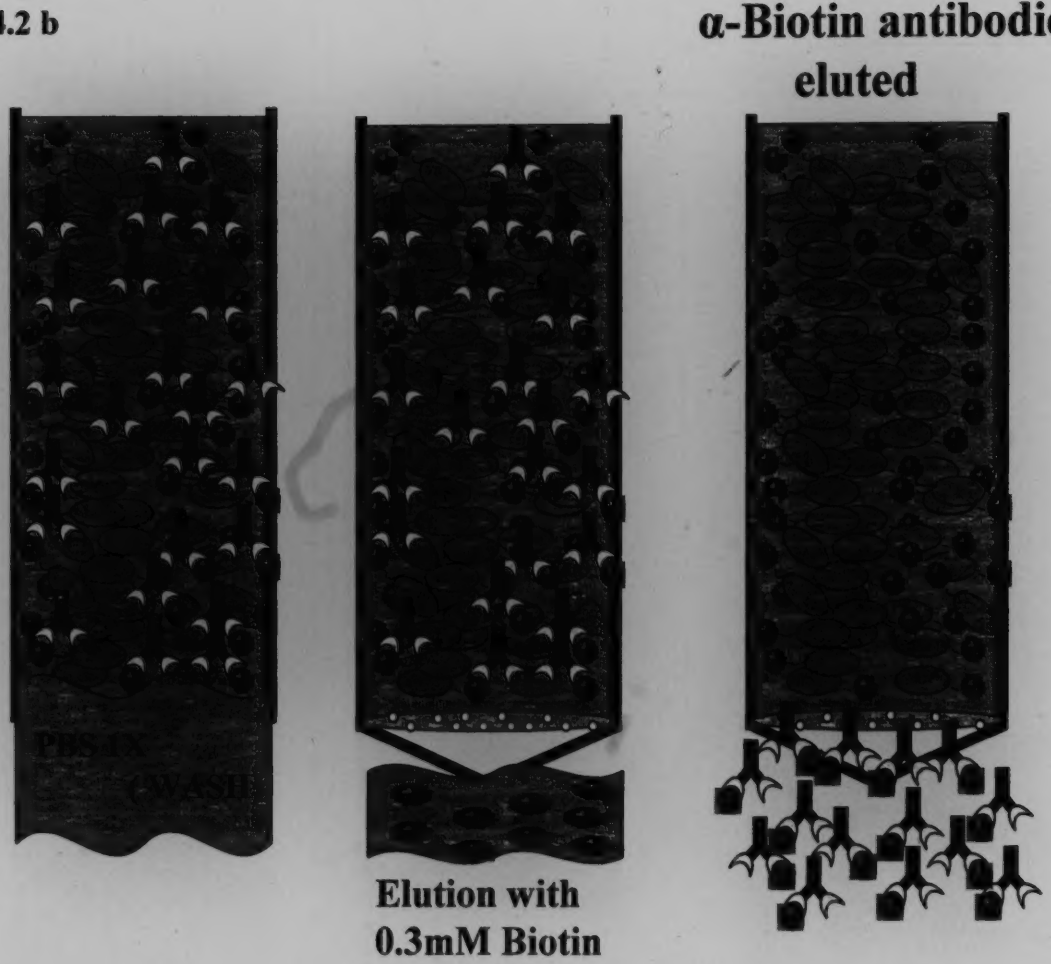

α-Biotin antibodies
eluted
PBS 1X
(WASH)
Elution with
0.3mM Biotin

Figure 4.3 a

Schematics of Mimetic Blue affinity purification technique

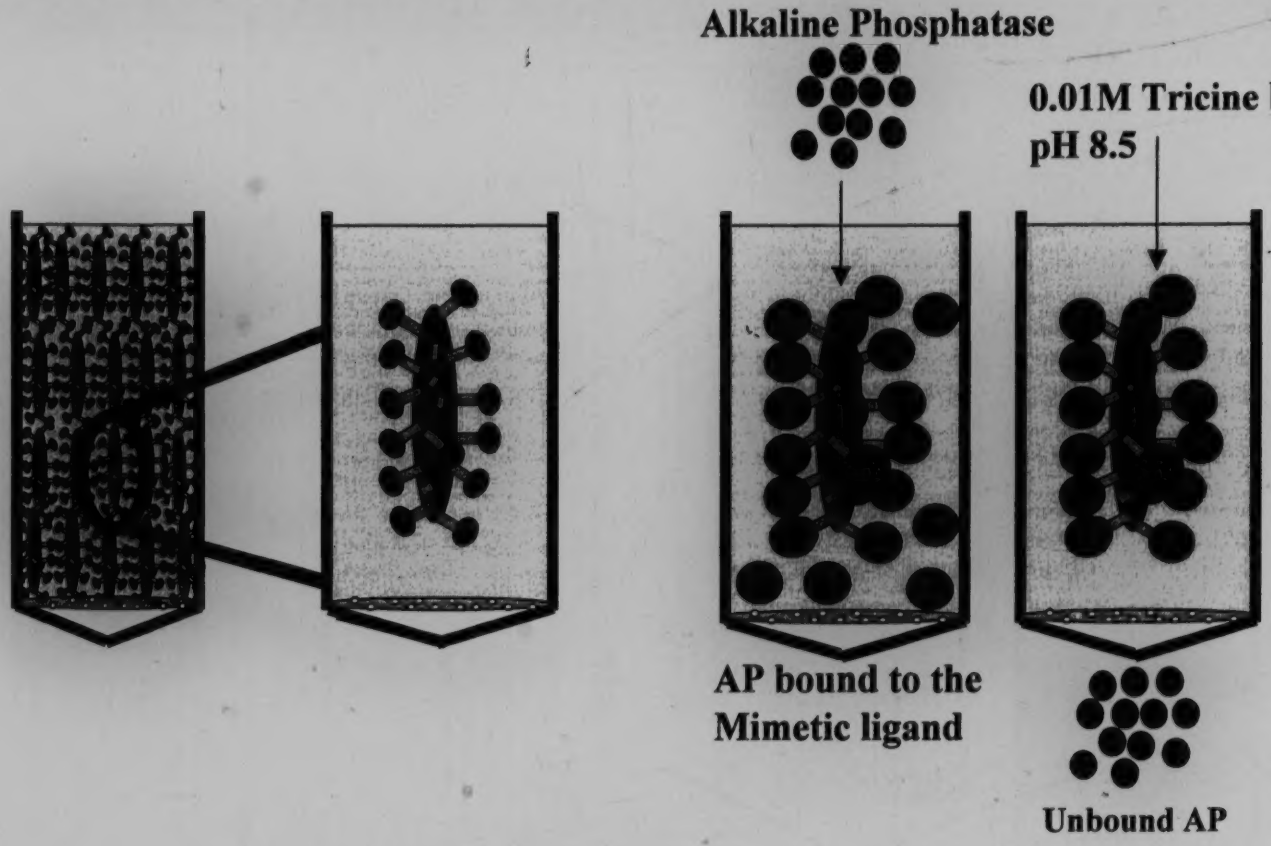
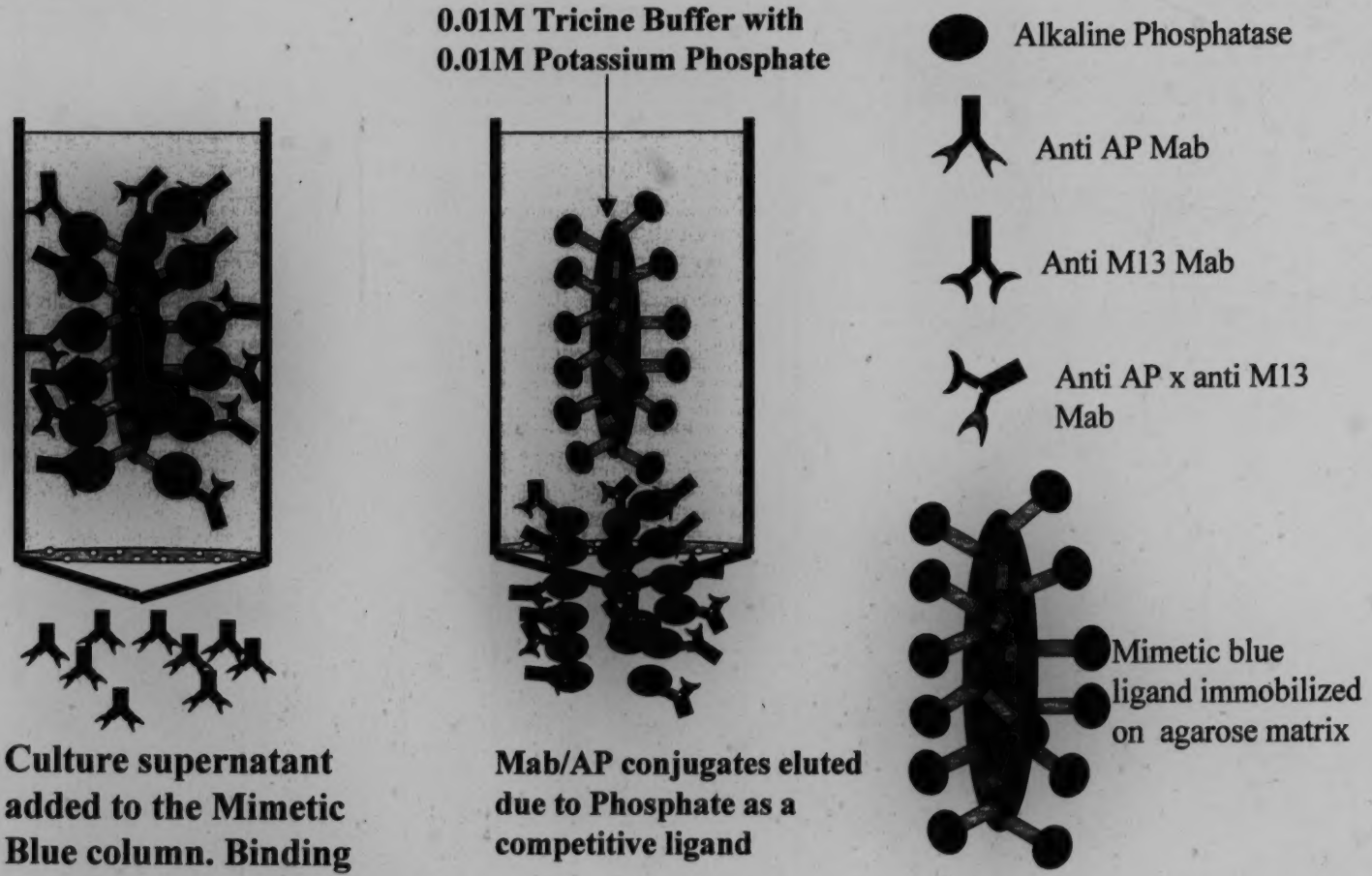


Figure 4.3 b


0.01M Tricine Buffer with
0.01M Potassium Phosphate
Alkaline Phosphatase
Anti AP Mab
Anti M13 Mab
Anti AP x anti M13 Mab
Mimetic blue ligand immobilized on agarose matrix
Culture supernatant added to the Mimetic Blue column. Binding of only the α AP Mabs and bs Mabs are depicted
Mab/AP conjugates eluted due to Phosphate as a competitive ligand

4.3 Results and Discussion

4.3.1 Protein-G Purification

The Mabs, A4A1, 11D2, 5B4D6, HB 191 and FMC-63 were mouse IgG class secreting hybridomas, and hence they were purified on the Protein-G affinity column. With the use of fetal bovine serum, irrelevant serum-derived antibodies and proteins would also be co-purified with protein-G, which is a group specific affinity matrix. Bovine serum supplemented media (at 10% FBS) contains less than 0.6% of bovine IgG. A significant problem with Protein-G adsorbents is that they avidly bind bovine IgG, leading to some contamination of the desired monoclonal IgG (Maiorella et al., 1990). Trebak et al., 1999 have reported that the Mabs obtained from serum-supplemented cultures contain not more than 0.6% of bovine IgG contamination. Serum-supplemented cell culture media typically provides higher productivity of the cell line. The prescence of serum is therefore beneficial for cell line productivity and stability (Maiorella et al., 1993).

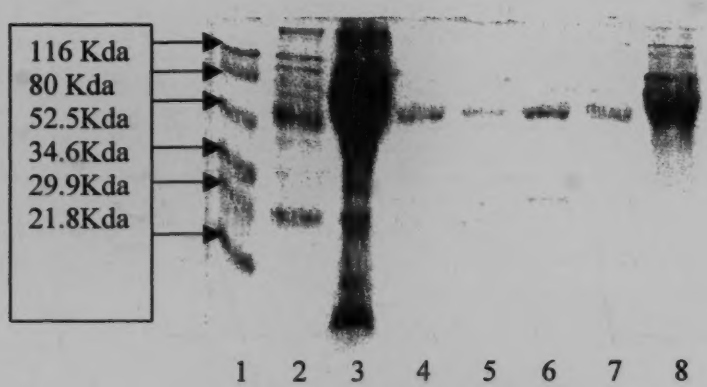


Figure 4.4 : Purified Mabs study
Lane 1-Marker; Lane 2-IgG; Lane 3-Mouse serum; Lane 4-A4A1; Lane 5-5B4D6; Lane 6-11D2; Lane 7-OKT3; Lane 8- FBS.

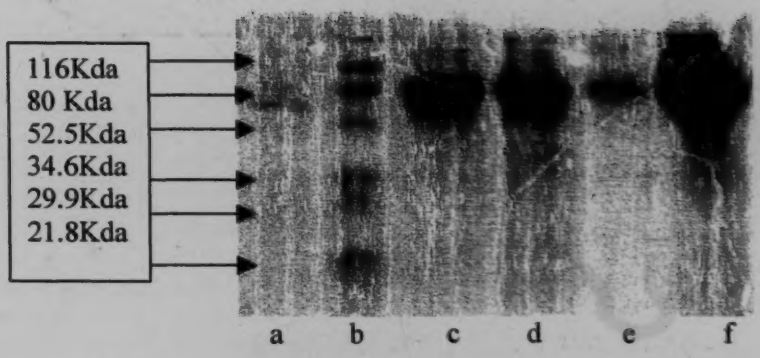
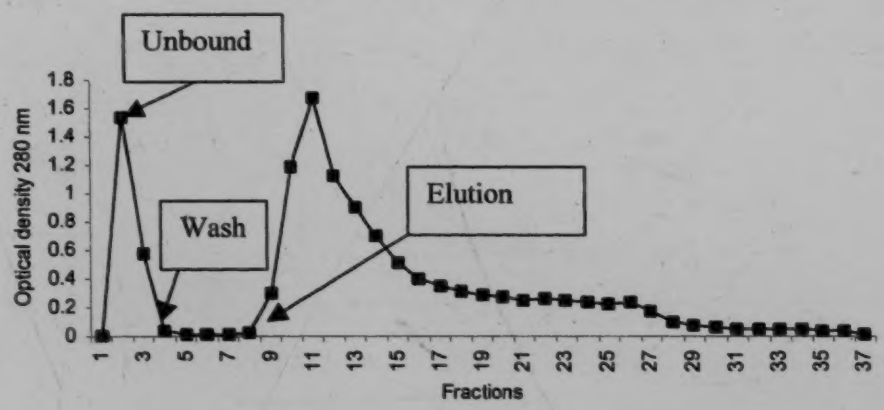


Figure 4.5 Unbound culture supernatant study

Lane a-IgG; Lane b-Marker; Lane c-11D2 unbound; Lane d-5B4D6 unbound ; Lane e-A4A1 unbound; Lane f- FBS.

From the above figures 4.4 & 4.5, the SDS-PAGE results show that there is no light chain in the unbound samples and the band at the 65 Kda range is due to the FBS in the culture supernatant. Whereas in the eluted fractions, there are bands at the 25 Kda and 50 Kda from the light chain and heavy chain of the Mabs respectively. From this it is evident that the Mabs are effectively purified on the protein-G column and retained on the column and subsequently obtained in the elution process.

Figure 4.6 UV Profile of Protein-G Purification of Mab

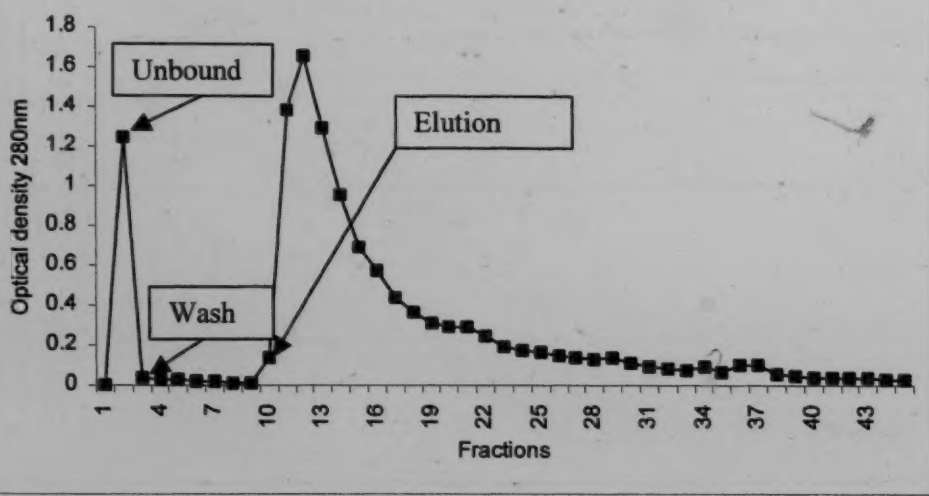


The study of the unbound fractions with the SDS PAGE figure 4.5, and eluted fractions in figure 4.4 revealed that the unbound fractions of the purification were not containing any fractions of the antibody. This experiment has confirmed that the protein-G was effectively binding the antibody in the culture supernatant. When the unbound pool was repurified, the recovery was mostly 50-100 μg for a volume of 2L unbound culture supernatant. This downstream loss is, hence negligible. One mL of Protein-G slurry can bind 25mg of the IgG, the efficiency of binding depends upon the slow flow rate with the pH at 7.0-7.5. Figure 4.6 shows a typical Protein-G purification profile.

4.3.2 Biotin affinity Purification

This purification technique has been used to purify α biotin Mab-P54 and is an example of a ligand specific affinity chromatography. The typical purification profile is shown in the figure 4.7. Unlike the group specific affinity chromatography with protein-G as described above, this method would not be expected to bind any contaminating trace bovine IgG.

Figure 4.7 UV Profile of Biotin Affinity Purification of Mab

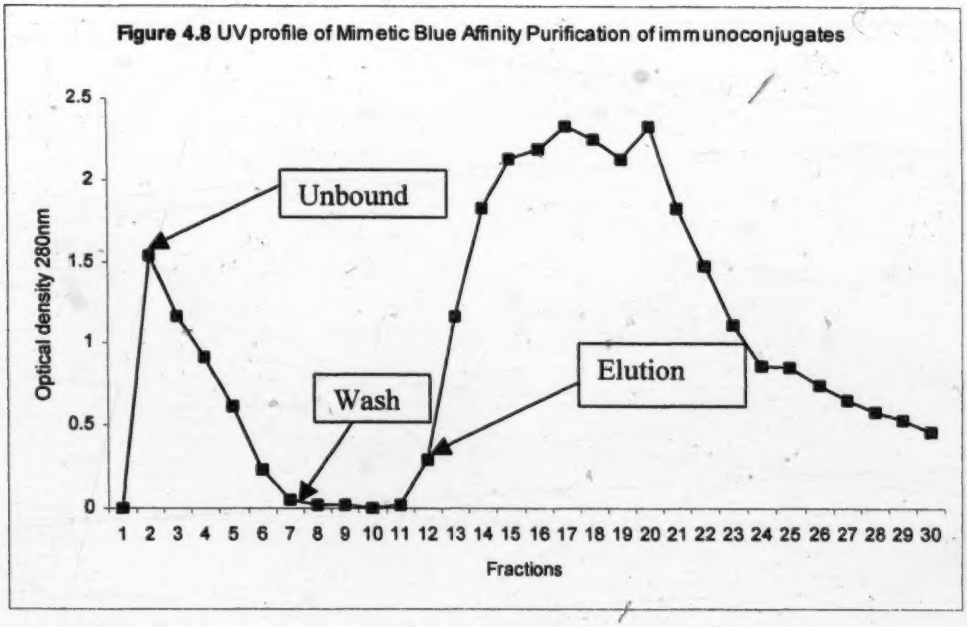


4.3.3 Mimetic blue –affinity purification

The mimetic Blue affinity chromatography procedure for purifying the P106 Mab is a unique variant of the affinity chromatography termed affinity co-chromatography. The mimetic column has affinity for alkaline phosphatase and the monospecific and bispecific antibody binds to the enzyme and hence is co-purified and eluted as antibody-enzyme conjugates. This provides a convenient method of generating immunoconjugates for diagnostic and therapeutic applications. The purification profile of this mimetic blue affinity purification is shown in the figure 4.8.

Enzymes such as alkaline phosphatase when conjugated to anti-cancer antibodies can be used potentially to convert a prodrug into an active drug near tumor cells (P.D.Senter1990). Purification of enzyme-antibody conjugates is generally accompanied by gel filtration chromatography (E.Harlow et.al 1988) and polymer precipitation (A. Polson et al, 1964). However, these methods lack the specificity to efficiently remove the free enzyme label, free antibody molecules and inactivated conjugates. Methods generally employed to purify bispecific Mabs include ion-exchange chromatography (M.R Suresh et.al 1986) or affinity chromatography on an antigen-immobilized column (P.J Kuppen et.al 1993). The former method lacks specificity and the latter one normally incorporates elution conditions that are harsh to the antibodies purified. Biospecific and pseudospecific mimetic ligands are being extensively used in the purification of the antibodies coupled to an enzyme against the mimetic ligand (NM Linder et.al.,1989).

Figure 4.8 UV profile of Mimetic Blue Affinity Purification of immunoconjugates



4.4 Conclusions

The affinity purification is the fastest and effective method of downstream processing to recover most of the Mabs from culture supernatant. Since affinity chromatography is more specific, the possibility of extraneous proteins being copurified are low. However, in the case of Protein G purification from culture fluids where fetal bovine serum is used, some amounts of bovine IgG is copurified with the desired Mabs. To overcome this problem, the Sterogene company has recently developed a IgG affinity slurry specially designed for the purification of the Mabs by its brand name QuickMAB™.

Different batches of ascities fluid and monoclonal antibody supernatant can vary widely in the amount of antibody they contain. Generally, 1mL of ascities should yield 1 to 4 mg of purified antibody and 1mL of Mab supernatant should yield 0.5 to 50 ug of purified product. When hybridomas are grown in bioreactors, higher yields can be obtained. However, yields significantly lower than these should be a warning that the antibody-producing hybridomas are likely being overgrown by non-producers and may warrant a recloning step. This can be remedied by returning to an earlier freeze of cells, or by recloning and rescreening the hybridoma.

Final Conclusions

- Ascities method of Mab production can be replaced with the in-*vitro* methods, taking into consideration the ethical concerns, minimal labor and cost effectiveness.

- Cell culture supplements such as oxygen carrier enhancers can increase the antibody production as shown in my experiments with PFCs.

- Gas permeable bags such as the I-Mab bags are ideal for the lab scale production of Mabs.They are scaleable, which would be economically beneficial and more humane method of preparing Mab.

- The production of Mabs by the cells depends on the nutritional medium, as was our experience with the various media. DMEM would be affordable and ideal for production of Mabs.

- The combination of the immunochemicals such as the goat anti-mouse IgG Fc on the solid phase and the goat anti-mouse IgG HRPO as the reporter enzyme has resulted in a ELISA format that can detect the mouse IgG concentrations without the interference of bovine IgG in the assays. This format was applied to detect the antibody secretion levels in the cell culture supernatants.

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